Development of an oral vaccine against the ostrich-specific mycoplasma, *Mycoplasma struthionis*

by
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Declaration

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March 2013

Abstract

The ostrich-specific mycoplasmas *Mycoplasma struthionis, Ms02* and *Mycoplasma nasistruthionis*, are associated with respiratory disease in ostriches, which is threatening the South African ostrich industry. Antibiotics are available to manage *Mycoplasma* infections, but a need to prevent infections led to an investigation into the development of vaccines against the ostrich-specific mycoplasma. After commercially available poultry mycoplasma vaccines proved to be unsuccessful in protecting ostriches against ostrich mycoplasma infections, the genome of *M. struthionis* was analysed and the *OppA* gene identified as a good vaccine candidate. The gene was isolated and used to develop the pCI-neo, VR1012 and VR1020 naked DNA vaccines. *M. struthionis* infects the respiratory tract of ostriches, therefore a vaccine that results in mucosal immunity is required. The use of a bacterial vector for DNA vaccines has been shown to elicit both a humoral and mucosal immune response. *Salmonella enterica* serovar *typhimurium* SL3261 was used to develop mucosal pCI-neo, VR1012 and VR1020 DNA vaccines. The pCI-neo mucosal DNA vaccine was found to be unstable *in vivo* and the stable mucosal VR1020 and VR1012 DNA vaccines were considered for subsequent vaccine trials. The tissue plasminogen activator (TSA) signal peptide found in the VR1020 plasmid to direct the secretion of the membrane protein, OppA, makes it a good candidate vaccine to compare against the naked DNA vaccine. A preliminary vaccine trial conducted with this vaccine was influenced by various factors including avian influenza and the statistical results proved to be invalid, but enzyme-linked immunosorbent assays (ELISAs) were developed for the successful measurement of the immune response of the ostriches. The dose of the mucosal vaccine administered in the preliminary trial might not have been enough to elicit an effective immune response in the ostriches. Different doses of the mucosal VR1012 and VR1020 DNA vaccines were therefore used in a second trial, but the trial was also influenced by a variety of factors. Even though the results of the vaccine trials were not successful, a few observations were made that could be used to improve future trials and reduce the effect of the factors on the vaccine trials, such as the effect of prior infection, as well as the stress on the ostriches.

Opsomming

Die volstruis-spesifieke mikoplasmas, *Mycoplasma struthionis, Ms02* en *Mycoplasma nasistruthionis*, word geassosieer met lugweg infeksies in voltruise, wat die Suid-Afrikaanse volstruisbedryf bedreig. Antibiotika is beskikbaar om mikoplasma infeksies te beveg, maar ‘n wyse om infeksie te voorkom word benodig. Dus is ‘n ondersoek geloods om effektiewe entstowwe te ontwikkel teen die volstruis mikoplasmas. Na die onsuksesvolle poging om pluimvee mikoplasma entstowwe, wat kommersiëel beskikbaar is, te gebruik om volstruise te beskerm teen volstruis mikoplasma infeksie, is die genoom van *M. struthionis* ondersoek en is die *OppA* geen geïdentifiseer as ‘n entstof kandidaat. Die geen is geïsoleer en gebruik om die pCI-neo, VR1012 en VR1020 deoksiribonukleïensuur (DNS) entstowwe te ontwikkel. *M. struthionis* veroorsaak infeksie in die respiratoriese kanaal van volstruise, daarom moet die entstof lei tot ‘n mukosale immuunrespons. Omrede daar al bewys is dat ‘n bakteriële vektor, as draer van ‘n DNS entstof, kan lei tot ‘n humorale sowel as ‘n mukosale immuunrespons, is *Salmonella enterica* serovar *typhimurium* SL3261 gebruik om mukosale pCI-neo, VR1012 en VR1020 DNS entstowwe te ontwikkel. *In vivo* was die mukosale pCI-neo DNS entstof onstabiel. Die stabiele mukosale VR1012 en VR1020 DNS entstowwe is gebruik vir die daaropvolgende entstof proewe. Die VR1020 plasmied bevat ‘n weefsel plasminogeen aktiveerder seinpeptied wat die sekresie van die membraan protein, OppA, lei, wat dit ‘n goeie kandidaat maak om te vergelyk met die skoon DNS entstof. ‘n Verskeindenheid faktore, insluitend voëlgriep, het die voorlopige entstof proef, wat uitgevoer is met hierdie entstof, beïnvloed en daar was te veel variasie in die statistiese resultate om tot ‘n gevolgtrekking oor elkeen van die entstowwe te kom. Die ensiem-gekoppelde immunosorberende toets wat ontwikkel is, was suskesvol daarin om die imuunrespons van die volstruis te meet. Die dosis van die mukosale entstof wat gebruik is in die voorlopige entstof proef, was dalk nie genoeg om ‘n doeltreffend imuunrespons in die volstruise te ontlok nie. Verskillende dosisse van die mukosale VR1012 en VR1020 DNS entstowwe is dus gebruik in ‘n tweede entstof proef, maar die entstof proef is ook beïnvloed deur verskeie faktore. Alhoewel die resultate van die entstof proewe nie na verwagting was nie, is observasies gemaak om toekomstige proewe te verbeter, soos om die effek van vorige infeksies, sowel as spanning op die volstruise te verminder.

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Abbreviations

ABC	ATP-binding cassette
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AI	Avian Influenza
ANOVA	Analysis of variance
APC	Antigen presenting cell
Aro	Auxotrophic mutant
ATP	Adenosine-5'-triphosphate
BGH	Bovine growth hormone
bp	Base pairs
CD	Cluster of differentiation
CMV-IE	Cytomegalovirus promoter immediate early gene promoter
CpG	Cytidine-phosphate-guanosine
CTL	Cytotoxic T-lymphocytes
DC	Dentritic cell
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
df	Degrees of freedom
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylene diamine tetra-acetic acid di-sodium salt
G + C	Guanine and Cytosine
GST	Glutathione s-transferase
i.d.	Intradermal injections
Ig	Immunoglobulin
i.m.	Intramuscular injections
IPTG	Isopropylthio-β-galactoside
LB	Luria broth
LPS	Lipopolysaccharide
LSD	Least significant difference
MAPS	Microbe-associated molecular patterns
Mϕ	Macrophage
M cells	Microfold cells

Mg-Bac	Mycoplasma Gallisepticum Bacterin
MHC	Major Histocompatibility Complex
MS	Mean of squares
Ms	*Mycoplasma struthionis*
MS-Bac	Mycoplasma Synoviae Bacterin
NALT	Nasopharynx-associated lymphoid tissue
Neo	Neomycin phosphotransferase
Nrdf	Ribonucleotide reductase
OD	Optical density
Opp	Oligopeptide permease
ori	Origin of replication
PAP	Peroxydase anti-peroxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGA	Polyglycolide
PLA	Polylactide
polyA	Polyadenylation
rRNA	Ribosomal ribonucleic acid
RIA	Radioimmunoassay
RSV	Rous sarcoma virus promoter
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp.	Species
spp.	Species (plural)
SS	Sum of squares
SV40	Simian vacuolating virus promoter
TAE	Tris-acetate EDTA
TLR	Toll-like receptor
tPA	Tissue plasminogen activator
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
XLD	Xylose lysine deoxycholate

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Chapter 1: Introduction

The South African ostriches industry is of significant importance to South Africa's economy, contributing over 1 billion rand to the country's economy annually as well as providing jobs to approximately 20 000 people (Anonymous May 2004, Van Helden *et al.* 2012). South Africa produces 70% of the world's ostrich products, making them good niche market products for the South African export market. The low cholesterol and fat levels in ostrich meat have made it a very popular alternative to red meat, arising from the recent trend to follow a healthier lifestyle, as well as Europe's declining confidence in the safety of red meat after various health scares. The increase in the demand for ostrich products has lead to a system of intensive rearing, resulting in a rise in diseases, in particular respiratory diseases.

Respiratory disease in ostriches can lead to production losses, carcass downgrading and increased susceptibility to secondary infection. Production losses of up to 30% have raised concerns of the economical impact of these diseases and for potential job losses. *Mycoplasmas* are among the pathogens that have been associated with respiratory infections in ostriches. Botes *et al.* (2005b) identified three ostrich mycoplasmas and provisionally described them as *Ms01*, *Ms02* and *Ms03*. *Ms01* and *Ms03* were subsequently officially described as *Mycoplasma struthionis* and *Mycoplasma nasistruthionis* (Langer 2009). Antibiotics, such as tylosin, are used to treat mycoplasma infections, and infection can be controlled through good biosecurity and farming practices. However, these methods only aim to treat or prevent the spread of infection; they are not effective at preventing infection. Vaccination against mycoplasma infections in chickens has been shown to be very effective in preventing infection thereby overcoming production losses. For this reason, an investigation has been launched in this laboratory since 2007 into the development of effective vaccination strategies against ostrich mycoplasma infections.

The first attempts by our laboratory included the use of commercially available poultry mycoplasma vaccines, which were ineffective at protecting the ostriches against the three ostrich specific mycoplasmas (Pretorius 2009). The second attempt included the design of a DNA vaccine effective against the ostrich-specific *Mycoplasma struthionis, Ms02* and *Mycoplasma nasistruthionis*. Pretorius (2009) identified a vaccine candidate gene for *Mycoplasma struthionis* using bioinformatic analyses which was then inserted into each of three plasmids, pCI-neo, VR1012 and VR1020. Brandt (2012) tested the efficacy of these naked DNA vaccines injected intramuscularly with inconclusive results, as these results were compromised by an outbreak of avian influenza (AI).

The purpose of this study was therefore to prepare mucosal vaccines as an alternative means to deliver the DNA vaccines with the following objectives:

* To identify a suitable bacterial carrier for the DNA vaccines
* To insert the previously prepared *Mycoplasma struthionis* DNA vaccines into the chosen bacterial carrier using electroporation to generate a mucosal DNA vaccine.
* To develop enzyme-linked immunosorbent assays (ELISAs) using recombinant *Mycoplasma struthionis* oligopeptide permease (OppA) protein as coating antigen for the analysis of the mucosal and serum immune responses of the ostriches against the vaccines.
* To develop ELISAs using *Salmonella enterica* serovar *typhimurium* SL3261 lipopolysaccharides (LPS) as coating antigens for the analysis of the mucosal and serum immune responses of the ostriches against the mucosal vaccines.
* To conduct a vaccination trial to test the ability of the mucosal DNA vaccine to elicit an immune response against the *Mycoplasma struthionis* OppA protein compared to the naked DNA vaccine as well as to test the immune response of the ostriches to the carrier bacteria.
* To conduct a vaccination trial to determine an effective dose for the mucosal DNA vaccine needed to elicit an immune response against the *Mycoplasma struthionis* OppA protein.

In Chapter 2 of this thesis the general characteristics of mycoplasmas, their pathogenesis and host distribution will be discussed, followed by a short description of the ostrich-specific mycoplasmas. A discussion on DNA vaccines with specific emphasis on bacterial carriers such as *Salmonella* mutants with which to deliver the DNA vaccines and the immune response elicited when these carriers are used follows. The insertion of the naked DNA vaccines against *Mycoplasma struthionis* into a chosen bacterial carrier is described in Chapter 3. A preliminary vaccine trial to compare the naked DNA vaccine to the mucosal DNA vaccine is described in Chapter 4, including the ELISAs used to test the humoral and mucosal immune response of the ostriches against the chosen *Mycoplasma struthionis* antigen and the carrier bacteria. The results of a further vaccine trial conducted to determine an effective dose for the mucosal vaccine that was needed to elicit an immune response in ostriches is described in Chapter 5. A conclusion and the future perspectives are given in Chapter 6, followed by the reference list. The thesis is concluded with addenda containing the results of the stability experiment of the plasmid vaccines in the bacterial carrier, as well as the raw data and graphs of both the preliminary vaccine trial, to compare the naked and mucosal DNA vaccines, and the vaccine trial to determine the effective dose of the mucosal vaccine.

Chapter 2: Literature review

2.1. Mycoplasmas

2.1.1. General characteristics and morphology

Mycoplasma is a genus in the family *Mycoplasmataceae*, which forms part of the order *Mycoplasmatales*, belonging to the class *Mollicutes* (Le Roux & Hoosen 2010). The name of the class *Mollicutes* comes from the Latin words 'mollis' meaning 'soft' and 'cutis' meaning 'skin' referring to the fact that prokaryotes in this class do not have a bacterial cell wall (Bradbury 2005). Other characteristics of free-living prokaryotes in this class are small genomes, with a low G + C content as well as some unusual nutritional requirements, such as the need for exogenous sterols (Kleven 2008, Weisburg *et al.* 1989).

Mycoplasmas are the smallest, self-replicating prokaryotes known to date, with sizes in the range of 0.2 to 0.7 µm (Le Roux & Hoosen 2010). Instead of the characteristic bacterial cell wall, mycoplasmas have a thin trilaminar cell membrane. Despite the lack of the bacterial cell wall, various mycoplasma species are found to have a wide range of shapes, ranging from spherical to flask-like and filamentous. Apart from the cell membrane, ribosomes and a circular double-stranded DNA molecule are the two other major cellular components found in mycoplasmas (Bradbury 2005). Due to these characteristics, mycoplasmas are resistant to most antibiotics, specifically the antibiotics that inhibit bacterial cell wall synthesis (Kleven 1998).

Mycoplasma genomes are very small and range in sizes from 0.58 - 1.38 Mbp with a low G + C content, ranging from 23% to 40% (Razin, Yogev & Naot 1998). It is thus not surprising that some of the first bacterial genomes that were sequenced fully came from this genus, such as the human pathogen *Mycoplasma genitalium* (Fraser *et al.* 1995). The genomes of a number of other mycoplasma species have also been sequenced and published, including the avian mycoplasmas *M. gallisepticum* (Papazisi *et al.* 2003) and *M. synoviae* (Vasconcelos *et al.* 2005). The small genome size of mycoplasmas is a result of reductive evolution from Gram-positive bacteria (Le Roux & Hoosen 2010, Razin, Yogev & Naot 1998), which resulted in a genome containing the minimum set of genes necessary for self-replication. An interesting change that also occurred in the mycoplasma genome during its evolution is the reassignment of the UGA stop codon to a tryptophan codon, while the other two stop codons remained stop codons. The simplicity of the genome does have its downsides in that the organism has a slower growth cycle and it is dependent on its host for most of its essential nutrients (Bradbury 2005).

2.1.2. Pathogenesis

Mycoplasmas enter the host mostly by inhalation, targeting the epithelial surfaces of the respiratory tract or the urogenital tract. Some mycoplasmas, such as *M. synoviae*, target the host joints. Apart from the ability to reach its targets sites, the gliding motility of some mycoplasmas, also aids in breaching the physical barriers of the host, such as the cilia and mucous layer lining the respiratory tract (Bradbury 2005, Kleven 2008).

One of the clinical signs observed during many mycoplasma infections are what are referred to as lesions, which is an injury in the epithelial tissue resulting in conditions such as sinusitis and catarrhal rhinitis (Botes *et al.* 2005a). The lesions might be an indirect result of the immune and inflammatory responses of the host to the pathogen (Bradbury 2005). Some studies suggest that mycoplasma infections occur mainly as a secondary infection in immune-compromised individuals, while other studies propose that some mycoplasmas, specifically avian mycoplasmas, appear to be part of a disease syndrome that includes various other bacterial species (Bradbury 2005, Kleven 2008).

Mycoplasmas appear to evade the host immune system by antigenic variation, which is a major factor in mycoplasma infection (Kleven 1998). Various mechanisms are utilised to cause variation of expression, as well as structure, of various important proteins, especially the proteins found on the cell surface (Bradbury 2005). Some studies on mycoplasma infection have also shown that other pathogens, such as *Escherichia coli* (*E. coli),* aid mycoplasmas in a synergistic manner to invade the host tissue and cause infection (Bradbury 2005, Kleven 2008).

An important step in mycoplasma infection is attachment to the target tissue. Most mycoplasmas accomplish adhesion with cell membrane-bound components, such as tip structures by *M. pneumonia, M. genitalium* and *M. gallisepticum* (Hu *et al.* 1987). Adhesin proteins mediate the attachment of mycoplasmas to host cells. The adhesin proteins include the most studied P1 protein of *M. pneumonia* (Razin & Jacobs 1992), the GapA protein of *M. gallisepticum* (Papazisi *et al.* 2003) and MgPa protein of *M. genitalium* (Le Roux & Hoosen 2010, Hu *et al.* 1987). The latter two both resemble the P1 protein of *M. pneumonia* (Razin, Yogev & Naot 1998). The P97 protein of *M. hyopneumoniae* (Zhang, Young & Ross 1995) showed no similarity to the P1 protein and might be a distinct mycoplasma adhesin. Henrich *et al.* (1993) studied adherence of *M. hominis* to its host tissue and identified the two surface-localized polypeptides, P50 and P100, as adhesins. The P100 polypeptide forms the substrate-binding domain of the oligopeptide permease (Opp) system named OppA (Henrich *et al.* 1999). The oligopeptide permease system is an ATP-binding cassette (ABC transporter) that uses energy from ATP hydrolysis to pump specific compounds across the biological membrane. The different components of this system are shown in figure 2.1 (Davidson &

Maloney 2007). Hopfe, Dahlmanns & Henrich (2011) discovered that the cytoadherence property of the OppA protein is dependent on its ATPase activity in the Opp system.

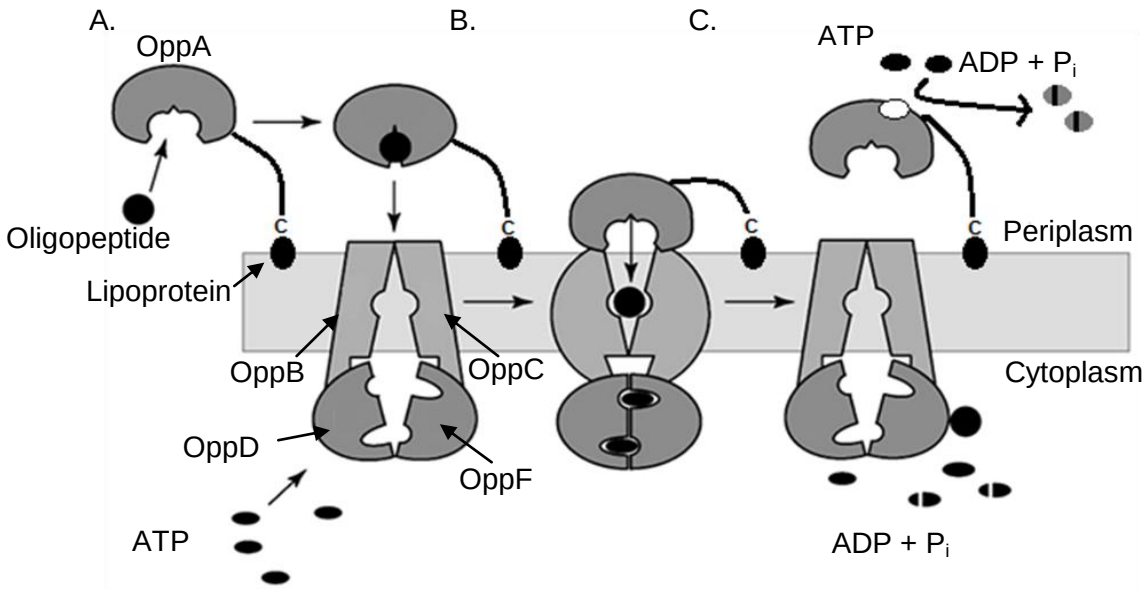

Figure 2.1: Oligopeptide permease (Opp) system: A. An oligopeptide binds to the substrate-binding domain (OppA). OppA transports the oligopeptide to the hydrophobic transmembrane domain (OppBC). B. The interaction of OppA with OppBC along with ATP binding at the hydrophilic ATP-binding domains (OppDF) causes i) ATPase activity activation upon closure of OppDF ii) a conformational change in OppBC exposing an oligopeptide binding site and iii) OppA to open and release the oligopeptide for binding to OppBC. C. After ATP hydrolysis on both OppA and OppDF, OppBC undergoes another conformational change, releasing the oligopeptide into the cytoplasm. Adapted from Davidson & Maloney (2007).

2.1.3. Host distribution

Mycoplasmas colonise a wide range of plant, mammal, insect, reptilian and avian species as commensals, but a few mycoplasma species are pathogenic and can lead to disease in their hosts (Razin, Yogev & Naot 1998, Volokhov *et al.* 2011). Apart from being mostly host-specific, mycoplasmas appear to be organ and tissue specific as well, mostly colonising the mucosal surface found in the respiratory and urogenital tract. Apart from these colonisation sites, some mycoplasmas can colonise the alimentary canal, eyes, joints and mammary glands (Vasconcelos *et al.* 2005). The host- and tissue-specificity of mycoplasmas may be attributed to their unusual nutritional requirements, such as cholesterol (Kleven 1998).

Domestic poultry infections have been the focus of most of the studies on avian mycoplasma infections, with *M. gallisepticum, M. synoviae, M. iowae* and *M. meleagridis* the four major pathogens associated with poultry mycoplasma infection. *M. gallisepticum* and *M. synoviae* infect chickens and turkeys, while *M. iowae* and *M. meleagridis* mostly only infect turkeys (Bradbury 2005). Botes *et al.* (2005b) studied mycoplasma infections in ostriches and identified three different mycoplasmas species that infect ostriches in South Africa using 16S rRNA gene sequencing, and

provisionally named them *Mycoplasma struthionis 01* (*Ms01*), *Mycoplasma struthionis 02 (Ms02)*, and *Mycoplasma struthioniss 03 (Ms03)* after their host *Struthio camelus*; the ostrich.

2.1.4. Ostrich-specific mycoplasmas

General characteristics and phylogeny

Botes *et al.* (2005b) tested samples from the main ostrich producing areas in South Africa. Apart from the identification of three novel mycoplasma species, Botes *et al.* (2005b) also determined that these three mycoplasmas did not infect poultry in close proximity to the infected ostriches, and are thus host-specific to ostriches. Langer (2009) isolated *Ms01* and *Ms03* from samples collected from ostriches in Namibia in an attempt to classify these species and described them as *Mycoplasma struthionis* sp. nov. and *Mycoplasma nasistruthionis* sp. nov. Langer (2009) found some similarities but also differences between these two species. On agar both species have a fried-egg appearance and are non-motile, they are chemo-organotrophs and require serum or cholesterol for growth (Langer 2009).

Mycoplasma struthionis, isolated from the lower respiratory tract, therefore replaces the provisional naming of ostrich-specific mycoplasma *Ms01*. These mycoplasmas form trunk-like structures and as can be seen in figure 2.2A, the cells do not aggregate. *Mycoplasma nasistruthionis*, isolated from the upper respiratory tract, replaces the provisional naming of the ostrich-specific mycoplasma *Ms03*. As seen in figure 2.2B the cells of this mycoplasma do aggregate (Langer 2009). To date ostrich-specific mycoplasma *Ms02* has not been described.

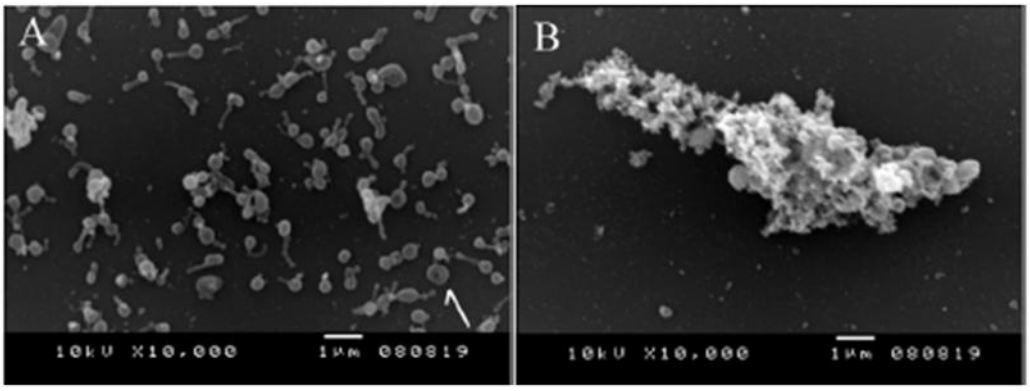

Figure 2.2: Scanning electron micrographs, taken from Langer (2009), of A. *Mycoplasma struthionis* sp. nov. and B. *Mycoplasma nasistruthionis* sp. nov. (Langer, Stefan 2009)

A phylogenetic study of the three mycoplasmas (figure 2.3) shows that *M. struthionis* (*Ms01*) falls in the same clade as *Mycoplasma falconis*, distinct from *Ms02* and *Mycoplasma nasistruthionis* (*Ms03*) that falls in the same clade as *Mycoplasma synoviae* (Botes *et al.* 2005b). Due to the close relationship, Botes *et al.* (2005b) proposed that the previous identification of *Mycoplasma synoviae* in ostriches (Verwoerd 2000) might have rather been *Ms02* or *Mycoplasma nasistruthionis* (*Ms03*).

Prevention, treatment and control

Mycoplasma infections in ostriches are associated with respiratory disease. As in mycoplasma infections in many other hosts, mycoplasma infections in ostriches are associated with co-infections with other bacterial pathogens (Botes *et al.* 2005a). Among the clinical signs observed are swelling of the sinuses, foaming of the eyes, a rattling sound in the throat as well as a watery discharge from the nostrils. These symptoms result in a reduction in growth rate, which delays slaughter age, as well as carcass downgrading and susceptibility to secondary infections resulting in stock and production losses (Olivier 2006).

Methods of control of mycoplasma infection in avian species include good biosecurity practices and good farming practices, but these methods are only partially effective. Regular monitoring of feed and water quality, weight increase, stress conditions and symptoms can aid in the management of mycoplasma infection (Olivier 2006). Some ostriches are mycoplasma carriers, showing no symptoms, and are only partially responsive to the treatments available, making it difficult to control mycoplasma infections on ostrich farms.

Mycoplasmas can be treated with antibiotics, but are resistant to conventional antibiotics that prevent cell wall formation, such as penicillin and cephalosporin (Kleven 1998). Among the antibiotics that mycoplasmas are sensitive to are tetracycline and macrolides that target protein synthesis and fluoroquinolones that inhibit DNA replication and transcription. In the South African ostrich industry the tetracyclines, oxytetracycline, doxycycline, chlortetracycline; the macrolide, tylosin; and the fluoroquinolone, advocin are used against mycoplasma infections (Olivier 2006). Even though these antibiotics are effective against the symptoms of mycoplasma infection, they only aid in the management and do not lead to the total eradication of mycoplasma infections. The use of antibiotics can lead to antibiotic resistance and antibiotics are not totally effective and expensive, and there is therefore an urgent need for an effective means to prevent mycoplasma infections in ostriches.

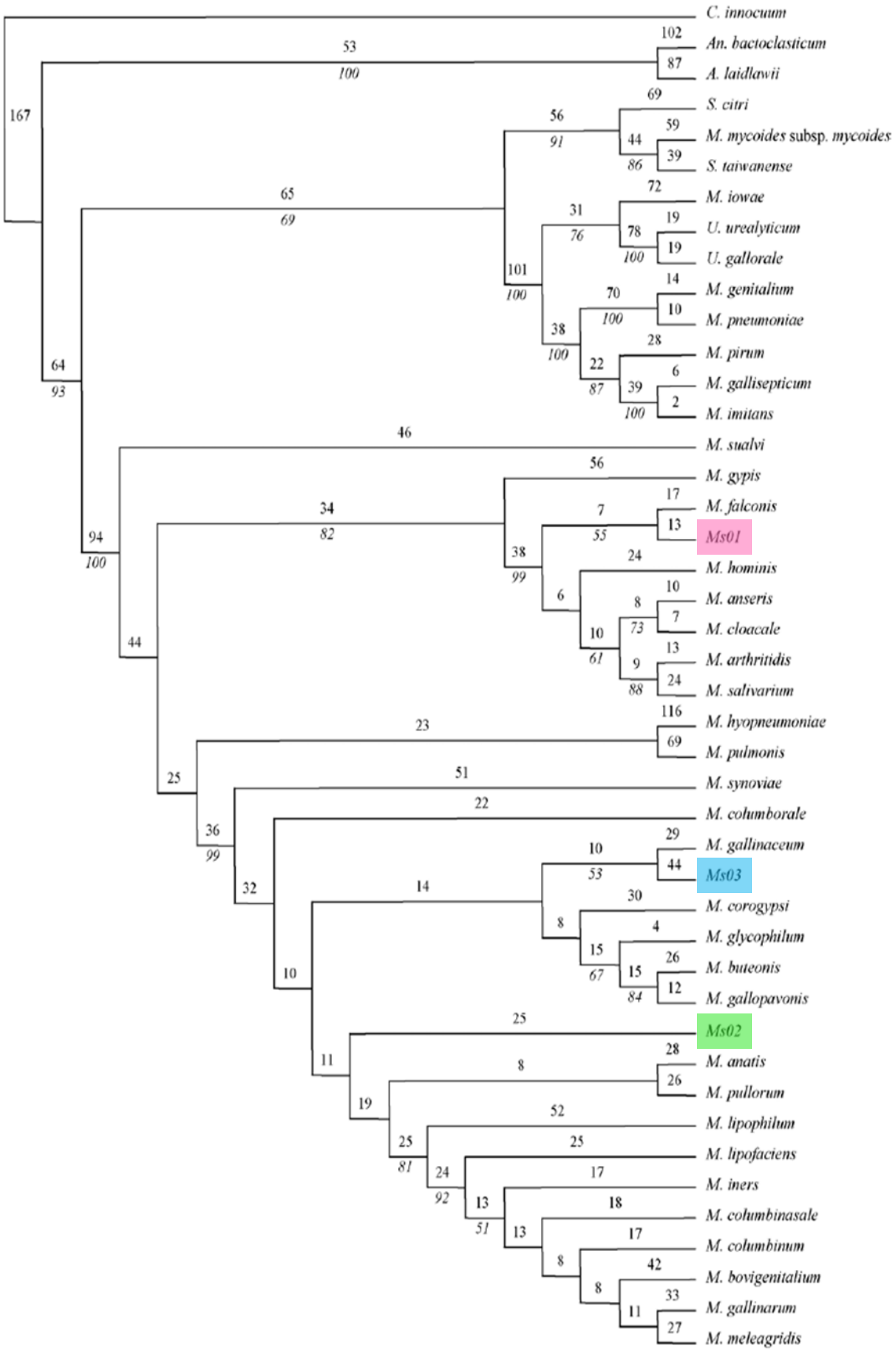

Figure 2.3: Phylogenetic relationships of the three ostrich-specific mycoplasma species to other mycoplasmas using 16S rRNA sequences. One of the shortest trees from the parsimony analysis is shown with the branch lengths indicated above branches and the bootstrap values below the branches. Branches with less than 50% bootstrap support are not shown (Botes *et al.* 2005b)

2.2. Vaccines

2.2.1. A short history of vaccines

Vaccines are biological agents used to improve immunity against a specific disease. The word vaccine originated from the latin word for cowpox disease, *Varioloe Vaccinae*, which was the first vaccine used in the Western World. Prior to this variolation, the smallpox virus itself was used to inoculate people, which was common practise in China. In the 18th century, Edward Jenner showed that cowpox could be used to prevent smallpox. Variolations were able to reduce the mortality rate as well as the development of scarring upon natural infection, but the use of the cowpox vaccine was a safer method. In the 19th century, Louis Pasteur found a way to attenuate organisms in the laboratory and developed vaccines against anthrax and rabies (Plotkin 2011). The chemical inactivation of a *Salmonella* sp. by Salmon and Smit in 1886 opened other possibilities that led to the development of the killed vaccines that were effective against cholera, typhoid and the plague towards the end of the 19th century (Plotkin 2011). Albert Calmette and Camille Guérin used *Mycobacterium bovis* to develop the live attenuated Bacille Calemette Guérin vaccine that was effective against tuberculosis in the early 20th century. Another important vaccine developed during this period was the live attenuated vaccine effective against yellow fever by Max Theiler in 1937 (Plotkin 2011).

2.2.2. Types of vaccines

At the start of the 21st century, the three main types of vaccines in use were live attenuated, killed and subunit vaccines (Alpar, Papanicolaou & Bramwell 2005). Live attenuated vaccines are mutants of the causative pathogens compromised in their ability to cause disease in the host, and include examples such as the vaccines for measles, yellow fever and mumps (Mäkelä 2006). The selected mutants are still immunogenic and can protect the host against infection from the wild type pathogen, but this is also a safety concern, as there remains the possibility of reversion to the pathogenic form (Alpar, Papanicolaou & Bramwell 2005). Killed vaccines are inactivated whole organisms and an example is the pertussis vaccine (Mäkelä 2006). Major safety concerns associated with killed vaccines include the possibility of incomplete activation, variable potency and contamination as was observed with the inactivated polio vaccine created by Salk in 1955, and adverse reactions, such as local inflammation and seizures caused by the pertussis vaccine. Subunit vaccines are vaccines where only a part of the pathogen is used and include the tetanus and diphtheria vaccines (Alpar, Papanicolaou & Bramwell 2005). Subunit vaccines are produced rapidly in cultured microorganisms, such as *E. coli*, at lower cost (Alpar, Papanicolaou & Bramwell 2005). This type of vaccine does have its flaws in that some have shown low immunogenicity and it is sometimes difficult if not impossible to identifiy the specific antigens that are capable of eliciting an immune response. The identification of candidate antigens as well as their presentation to the

immune system has become the main focus of current vaccine research (Alpar, Papanicolaou & Bramwell 2005).

Wolff *et al.* 1990 (Wolff *et al.* 1990) observed expression of a reporter transgene in transfected mouse cells after an injection of plasmid DNA. After Tang *et al.* (1992) introduced a plasmid containing a gene encoding a protein to the mouse skin and observed an immune response, he suggested that this method could be used as a genetic immunization against pathogens. Together this led to the discovery of DNA vaccines that can be produced at lower cost, with increased stability, higher purity and a lower risk of reversion (Alpar, Papanicolaou & Bramwell 2005). This vaccine type also has the advantage that only the antigen of interest is expressed and both antibody- and cell-mediated immune responses can be induced. There are, however, concerns associated with DNA vaccines, such as germ-line alterations and the induction of immune tolerance and autoimmunity (Alpar, Papanicolaou & Bramwell 2005). Despite these concerns numerous advantages of DNA vaccines make them a more attractive approach for vaccine design (Alpar, Papanicolaou & Bramwell 2005).

2.2.3. Mycoplasma vaccines

There have been a number of attempts to generate effective mycoplasma vaccines. Vaccines against the poultry mycoplasmas *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, include inactivated, oil emulsion bacterins and live attenuated vaccines (Kleven 1998). An immune response was elicited in ostriches againts two commercially available oil-emulsion poultry mycoplasma vaccines, Ms-Bac and Mg-Bac against *Mycoplasma synoviae* and *Mycoplasma gallisepticum*, respectively (van der Merwe 2006). Further studies showed that these vaccines were unable to protect the ostriches against ostrich-specific mycoplasma infection (Pretorius 2009). It was also found that cultivation of the ostrich-specific mycoplasmas is difficult, which makes it difficult to follow the traditional routes of vaccine development (Botes *et al.* 2008). The advantages of DNA vaccines over killed and live vaccines, has directed recent research on mycoplasma vaccines mainly to DNA vaccines. DNA vaccines generated against *Mycoplasma hyopneumoniae*, which causes enzootic pneumonia in pigs, have shown promising results with an immune response elicited in mice (Chen *et al.* 2003) and reduced infection in pigs (Fagan *et al.* 1996).

2.2.4. DNA vaccines

DNA vaccine design

The typical generation of a DNA vaccine is shown in figure 2.4. Firstly the genome of the pathogen is studied to identify a suitable vaccine candidate gene. This candidate gene should ideally encode an antigenic protein that can elicit a protective immune response (Doria-Rose, Haigwood 2003). Membrane proteins associated with cytadherance and transport are immunogenic and considered as good candidates for use in the development of DNA vaccines. Pretorius (2009) used next generation sequencing to sequence a major part of the genome of *Mycoplasma struthionis.* Through bioinformatic analysis of the contiguous sequences that were generated, the o*pp*A gene of *Mycoplasma struthionis* was identified by sequence homology to the o*ppA* gene of *Mycoplasma hominis* (Pretorius 2009). The o*ppA* gene is the substrate binding domain of oligopeptide permease system (figure 2.1) involved in host attachment and nutrient uptake, making it a good candidate gene for vaccine development (Garmory & Titball 2004, Tanabe *et al.* 2006).

The next step in developing a DNA vaccine is to insert the candidate gene into a suitable vaccine plasmid. A number of elements are required to construct a suitable plasmid that can be used as a vector for DNA vaccination. An origin of replication (ori), the sequence where replication is initiated, and a selection marker are both required to allow plasmid reproduction and the stable maintenance of the plasmid when grown in bacteria. An example of a suitable ori is the Co1EI gene of *E. coli* of the pUC plasmid, which is a high copy number ori, and increases the plasmid yield (Ertl & Thomsen 2003). An antibiotic resistance gene is used as the selection marker to ensure multiplication of plasmids containing the candidate gene. To direct expression of the candidate gene in a eukaryotic host a strong eukaryotic promoter and a eukaryotic polyadenylation (polyA) signal must be included. Eukaryotic promoters that mediate high expression levels include the Rous sarcoma virus promoter (RSV) and the Simian vacuolating virus promoter (SV40) (Donnelly *et al.* 1997). The cytomegalovirus promoter immediate early gene promoter (CMV-IE), along with the first intron from the IE1 gene (intronA), results in strong expression and is most often used (Ertl & Thomsen 2003). The polyA signals used most often are the polyA signal of SV40 and bovine growth hormone (BGH) to stabilize the mRNA transcript (Gurunathan, Klinman & Seder 2000). The promoter and polyA signal are separated by a multiple cloning site, which is used to insert the candidate gene using restriction sites (Ertl & Thomsen 2003). The candidate gene is then cloned into such a plasmid vector to generate the required DNA vaccine.

The vaccine plasmid into which the candidate gene is inserted is multiplied in bacteria, such as *E. coli*, to increase the number of plasmid copies. The DNA vaccine can then be isolated from the bacteria and purified for host immunization. The routes of administration mostly used to deliver DNA vaccines are intramuscular injections (i.m.) or intradermal injections (i.d.) with either a

hypodermic needle or a gene gun (Dufour 2001). After DNA immunization, either the somatic cells at the site of injection, myocytes in the case of i.m. injection, or professional antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages can be transfected. The transfected somatic cells express and secrete the antigen for presentation by professional APCs. The transfected APCs can either process and present the antigen itself or secrete the antigen for presentation by another APC. APCs then present the antigen on Major Histocompatibility Complex (MHC) class I and class II molecules as shown in figure 2.4B. MHC class I molecules present the antigen to the cytotoxic T-lymphocytes (CTLs) receptors and $CD8^+$ receptors resulting in a cellular immune response. The antigen presented on MHC class II molecules is presented to helper T-lymphocytes receptors and $CD4^+$ receptors. Helper T-lymphocytes promote a cellular immune response by aiding in the proliferation of activated CTLs. Helper T-lymphocytes also stimulate the proliferation of B cells to produce a humoral immune response (Gurunathan, Klinman & Seder 2000). DNA vaccines thus have the potential to elicit both a B- and a T-cell immune response.

Efforts to generate DNA vaccines against pathogens were effective in many mouse studies, but appeared to be less effective in humans and larger animals. The low potency was attributed to the low level of APC transfection achieved, which redirected research to improving methods of administration. Methods that have been used include electroporation after needle injection, jet ejection and topical application at mucosal sites. To elicit an effective immune response with these methods, large doses of plasmid DNA are required, also resulting in low potency in large animals. Research efforts then focused on effective modes of delivering the plasmid DNA to the immune system (Donnelly, Berry & Ulmer 2003). These efforts included adjuvants, such as liposomes and biodegradable polyester particles (Alpar, Papanicolaou & Bramwell 2005). Liposomes are bilayered phospholipid particles used to encapsulate both water-soluble and hydrophobic particles, but can be toxic and in biological environments tend to be unstable (Alpar, Papanicolaou & Bramwell 2005). Biodegradable polyester particles are polymers, such as polyglycolide (PGA) and polylactide (PLA), used to encapsulate particles (Alpar, Papanicolaou & Bramwell 2005). Although these particles have been used successfully to deliver protein antigens, they carry a slight negative charge that may lead to inefficient encapsulation of DNA plasmid vaccines (Alpar, Papanicolaou & Bramwell 2005). A method of delivery that shows promise is the use of bacterial vectors to deliver DNA vaccines, especially to elicit a mucosal immune response that would be required to create an effective vaccine against *Mycoplasma* spp. as described in the next section (Alpar, Papanicolaou & Bramwell 2005).

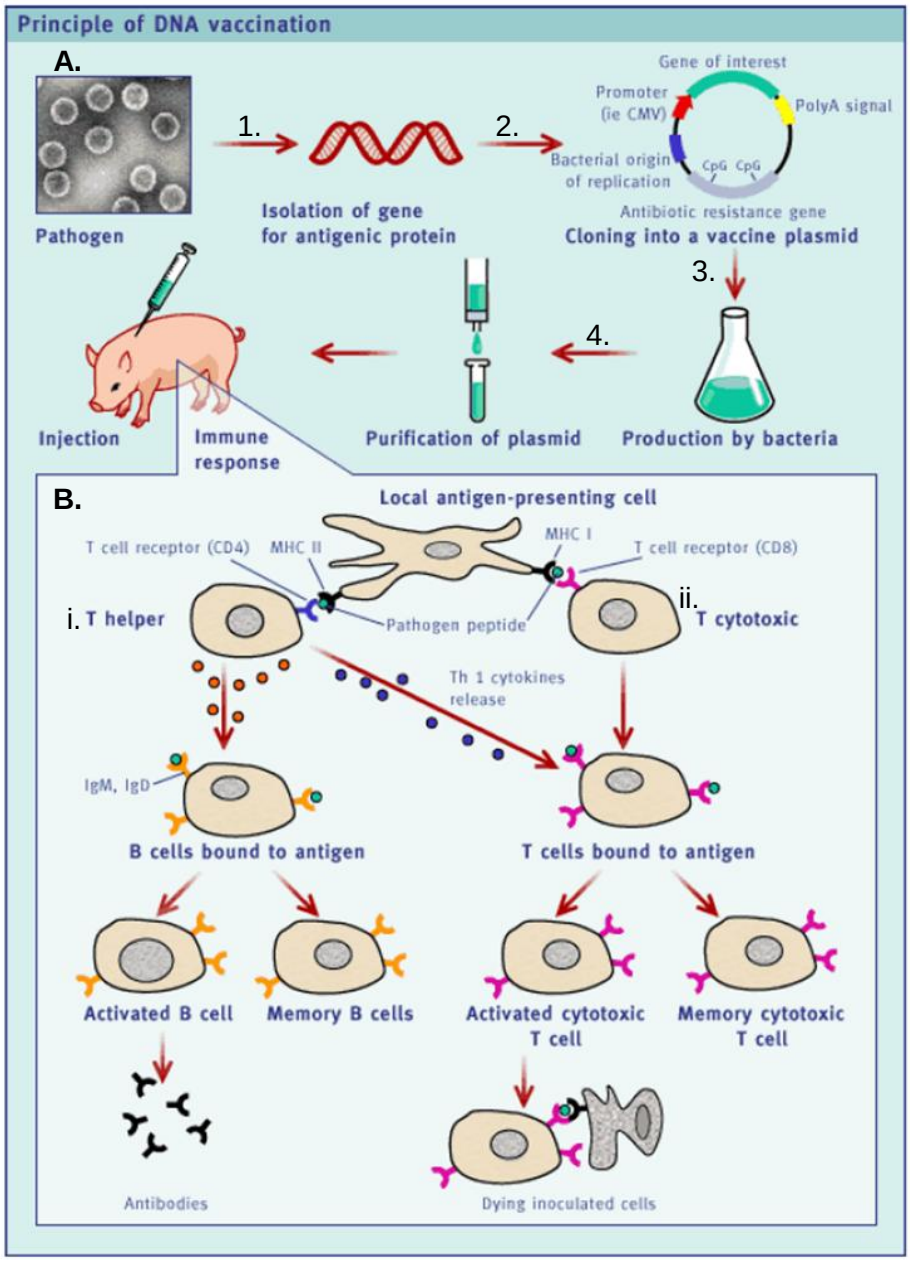

Figure 2.4: Graphic representation of DNA vaccine design and immune activation taken from (Dufour, V. 2001). **A.** DNA vaccine preparation: 1. The identification and isolation of a candidate gene. 2. Cloning of candidate gene into a suitable expression vector. 3. The amount of plasmids is increased by propagation in bacteria. 4. Isolation and purification of plasmids from the bacteria to immunize the host. **B.** Immune response elicited by DNA vaccine: After injection of the vaccine, the candidate gene is transcribed and translated in the nucleus and presented on MHC class I and II molecules. i. MHC class II molecules are found on APCs such as DCs and B cells and present the antigen to CD4+ receptors present on helper T-lymphocytes. I.m. injections primarily promotes the activation of a T-helper cell 1 (Th1) response, which aids in the proliferation of activated CTLs. A T-helper cell 2 (Th2) response can also occur and activates a humoral immune response by stimulation of B cell maturation. B cell maturation results in both the production of antibodies and development of memory B cells ii. MHC class I molecules are found on nucleated cells, thus excluding erythrocytes, and present the antigen to $CD8^+$ receptors on cytotoxic T-lymphocytes. Once activated the CTL can either induce apoptosis of cells displaying the antigen or activate macrophages and natural killer (NK) cells (Gurunathan, Klinman & Seder 2000).

2.2.5. DNA vaccines against the ostrich-specific mycoplasma, *Mycoplasma struthionis*

Pretorius (2009) isolated the *oppA* gene from *Mycoplasma struthionis* and inserted the gene into each of three plasmid vectors, pCI-neo, VR1012 and VR1020. All three of these plasmids have a CMV promoter promoting strong expression of the gene upstream of a universal vaccine candidate gene insertion site. Apart from the CMV promoter, the pCI-neo plasmid vector (figure 2.5) also has a phosphotransferase gene, which is a selectable marker for mammalian cells aiding in the selection of the stable transformants, a downstream SV40 polyA signal and the ampicillin resistance gene (Promega September 2009, Mortensen *et al.* 2001). The VR1012 plasmid vector (Vical Inc) is made up of intronA, the BGH polyA signal and contains a kanamycin resistance gene. The VR1020 plasmid vector (Vical Inc) also has a BGH polyA signal and kanamycin resistance gene, but differs from VR1012 with the presence of the upstream tissue plasminogen activator (tPA) signal peptide that aids in secretion of the protein after expression (Coban *et al.* 2005). In this way, Pretorius (2009) therefore generated three potential DNA vaccines for use against the ostrich-specific mycoplasma, *Mycoplasma struthionis*. The effectivity of these vaccines have not been conclusively determined.

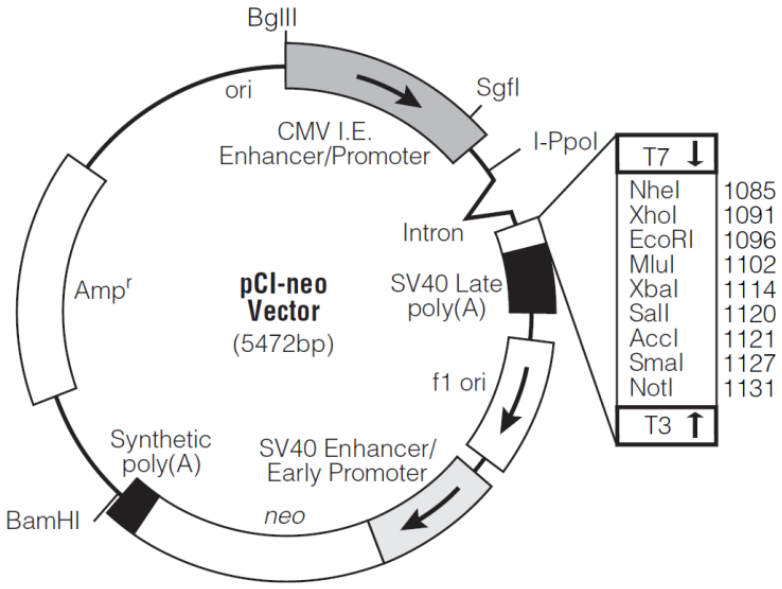

Figure 2.5: Circle map of the pCI-neo vector map. Neo = neomycin phospotransferase, CMV I.E. = cytomegalovirus immediate-early.

2.2.6. Bacterial carriers of DNA vaccines

Advantages and disadvantages of bacteria as DNA vaccines carriers

Apart from the use of bacteria to improve the DNA vaccine delivery to the host immune system, bacteria also add other advantages for their use as DNA vaccine carriers. The use of naked DNA as a vaccine requires prior plasmid amplification and purification, which can be labour intensive and plasmid degradation can occur during storage. However, a DNA vaccine plasmid can be

transformed into a bacterial vector with relative ease using methods such as electroporation or chemical methods. After transformation, only proliferation of the bacteria is required to produce the bacterial DNA vaccine, lowering the cost and labour necessary for mass production and large-scale immunization (Schoen *et al.* 2004).

Bacterial DNA carrier vaccines are commonly administered as oral vaccines, which reduces the time and labour required to immunize large groups of animals. Oral vaccination also reduces the amount of stress the host experiences which then reduces the risk of a compromised immune system (Qu *et al.* 2008). The use of naked DNA vaccines mostly stimulate a serum immune response, but bacterial DNA vaccines can elicit both a mucosal and serum immune response (Barry *et al.* 1996). Other advantages of bacterial DNA vaccines include their use to target specific sites for release of the DNA vaccine and the possibility that more than one DNA vaccine can be delivered per bacterial carrier (Barry *et al.* 1996, Pasetti *et al.* 1999).

A concern for the use of bacteria as vaccine carriers is the possibility of reversion to virulence, but this risk is lowered when the bacterial strain is attenuated with multiple deletions (Autenrieth & Schmidt 2000). Live viruses have also been considered as vaccine carriers, but the use of bacterial carriers has some advantages over viruses when infection by the vector occurs. If a host is immune compromised, the carrier vector can cause infection, but there are effective antibiotics available to clear up a bacterial infection, which is better than the treatments required for viral infection (Schoen *et al.* 2004)**.**

Use of bacteria as DNA vaccine carriers

Bacteria can be used to deliver DNA vaccines to the immune system either as recombinant vaccines, where the bacteria are used to express the antigen for presentation to the immune system, or as carrier, where the bacteria deliver the DNA plasmid vaccine to the immune system. Chen and co-workers (2006b) found that using *Salmonella typhimurium* only as a bacterial carrier of the antigen, NrdF, is more effective as an oral vaccine to induce an immune response than if *Salmonella enterica* serovar *typhimurium* is used to express the antigen (Fagan *et al.* 1997).

Intracellular bacteria are exploited as carriers of DNA vaccines for their ability to infect the gut mucosa successfully and to stimulate an immune response. The bacteria most often used are the extracellular bacteria, *Yersinia* spp.; the intracytosolic bacteria, *Listeria monocytogenes* and *Shigella* spp. and the intraphagosomal bacteria, *Salmonella* spp. (Pasetti *et al.* 1999, Sizemore, Branstrom & Sadoff 1997). Proliferation of the bacterium, Y*ersinia enterolitica*, occurs extracellularly once it crosses the mucosal barrier to the abdominal lymphatic tissue. Attenuation can diminish the resistance of this bacterium to phagocytosis allowing delivery of DNA vaccines. *Shigella* spp. cross the mucosa microfold cells (M cells) found in Peyer's patches and have the ability to invade macrophages and DC's. *L. monocytogenes*, is the only Gram-positive carrier strain

that has been used thus far. This organism has the advantage over Gram-negative bacteria in that it does not possess lipopolysaccharides, eliminating their potential toxic effects. *L. monocytogenes*, also has the ability to invade the APC's and multiply within the cell cytosol, but this organism can spread to other tissues by moving from one cell to another. Attenuated strains used can still enter the cell cytoplasm, but are hampered in their ability to spread to other cells. *Salmonella* spp., more specifically *Salmonella enterica* serovar *typhimurium* and *Salmonella enterica* serovar *typhi* also enter the lymphatic system through the M cells that line Peyer's patches and then induce their uptake by phagocytes. Attenuated *Salmonella* spp. can still induce uptake by the phagosome, but do not replicate, and release the DNA vaccine (Gurunathan, Klinman & Seder 2000).

2.2.7. *Salmonella* as carriers of DNA vaccines

Salmonella are Gram-negative bacteria in the order Enterobacteriales. These rod-shaped (figure 2.6) bacteria are facultative anaerobes and intracellular pathogens of both humans and animals. *Salmonella* are considered good candidates for DNA vaccine carriers due to their ability to invade phagocytes and proliferate without being phagocytosed, thereby evading the host immune system (Schoen *et al.* 2004, Turner, Carbone & Strugnell 1993). Numerous attenuated *Salmonella* spp. have been modified for use as carriers of DNA vaccines, some of which are listed in Table 2.1. Most of the studies listed were conducted in mice, but a few of the studies have shown that they can give protection to infection with virulent pathogens.

The *Salmonella enterica* serovar *typhimurium* strain SL3261 was used to carry a DNA vaccine against Feline Immunodeficiency Virus (FIV) that elicited an immune response in cats (Titball *et al.* 1997). In dogs, *Salmonella enterica* serovar *typhimurium* LVR01 was used as a DNA vaccine carrier to elicit an immune response against *Echinococcus granulosus* (Chabalgoity *et al.* 2000). It has been proposed that using a strain of *Salmonella* that naturally infects the host might elicit a better immune response as these strains are adapted for infection of the host of interest (Chabalgoity *et al.* 2000, Stabel *et al.* 1993). In pigs various *Salmonella* spp. have been used as carriers against various diseases, such as *Brucella abortus* (Stabel *et al.* 1993) and *Mycoplasma hyopneumoniae* (Fagan *et al.* 1997, Fagan *et al.* 2001,Fagan *et al.* 1997). In chickens *Salmonella* spp. have also shown promise as carriers of a DNA vaccine against *Eimeria tenella* (Pogonka *et al.* 2003). In the context of this study *Salmonella enterica* serovar *typhimurium* is a common pathogen found in ostriches (Verwoerd 2000).

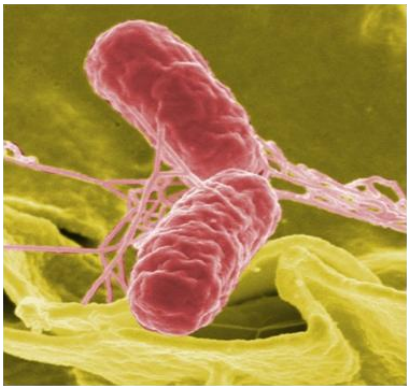

Figure 2.6: Scanning electron micrograph of *Salmonella typhimurium* taken from Rocky Mountain Laboratories (2006)

Salmonella enterica serovar *typhimurium* SL3261 is a non-reverting, highly attenuated *aroA* mutant generated by Hoiseth and Stocker (1981). This strain has a deletion in the *aroA* gene, with the result that it has become an auxotrophic organism with limited growth *in vivo* (Chen *et al.* 2006b, Fagan *et al.* 1997, Fagan *et al.* 2001). Auxotrophic mutations inhibit bacterial proliferation due to an inability to produce essential compounds (Raupach & Kaufmann 2001). *Salmonella enterica* serovar *typhimurium* SL3261 require the compounds p-aminobenzoic acid and 2,3-dihydroxybenzoate, not found in mammalian cells, for aromatic amino acid synthesis as well as for growth (Raupach, Kaufmann 2001). In spite of this, a mucosal as well as a humoral immune response can be elicited by such *aroA* mutants (Yang *et al.* 1990).The *aroA* mutants can invade, colonize and persist *in vivo*, but remain avirulent. This allows the bacterium to prime both cellular and humoral immunity by interaction with macrophages, B cells and T cells, making it a good candidate for use as a DNA vaccine carrier. These mutants have been shown to serve as effective carriers of DNA vaccines (Bao & Clements 1991, Chatfield *et al. 1993)*.

Immune response elicited by Salmonella *bacteria*

To determine the immune response elicited by *Salmonella* spp. various studies have been performed, but so far only a basic understanding of *Salmonella* infections has been established. The mucosal immune response elicit by bacteria after oral or intranasal application is shown in figure 2.7. (Kiyono & Fukuyama 2004). *Salmonella* spp., in most studies administered orally, attach to the epithelial layer of the intestinal tract with the aid of fimbral adhesions and invade the M cells that line the Peyer's patches in the intestine. M cells are specialized epithelial cells found in the Nasopharynx-associated lymphoid tissue (NALT) and Peyer's patch follicle; and transport microorganisms and other macromolecules across the epithelial layer to the cells of the immune system. M cells play an important role in stimulation of mucosal immunity. The bacterium then induces phagocytosis by APC's. APC's include cells such as DC's, macrophages (Mϕ) and B cells. DC's take up the bacterial antigens by endocytosis and macrophages phagocytose bacteria (Kiyono, Fukuyama 2004).

Table 2.1: Strains of attenuated *Salmonella* spp. used as vaccines carriers.

Bacteria	Mutant	Mutation	Strains	Organism (Disease)	Reference
Salmonella enterica serovar *typhimurium*	*aroA*	Impaired in their ability to produce aromatic amino acids required for *in vivo* replication	SL3261	*Yersinia pestis* (plague), Feline immunodeficiency virus, *Cryptosporidium parvum* (cryptosporidiosis), *Mycoplasma hyopneumoniae* (porcine enzootic pneumonia), *Eimeria tenella* (coccidiosis)	(Kiyono & Fukuyama 2004)
			SL7207	*Listeria monocytogenes* (listeriosis), H9 low-pathogenic avian influenza virus	(Bauer *et al.* 2005, Pan *et al.* 2009)
			SL7237	*Listeria monocytogenes* (listeriosis)	(Darji *et al.* 2000)
			CS332	*Mycoplasma hyopneumoniae* (porcine enzootic pneumonia)	(Chen *et al.* 2006a)
	aroC		LVR01	*Echinococcus granulosus* (hydatid disease)	(Chabalgoity *et al.* 2000)
	htrA	Mutation in a gene for a stress protein (heat shock protein)	C5htrA	*Eimeria tenella* (coccidiosis)	(Pogonka *et al.* 2003)
	Dam^{-} *phoP*$^{-}$	Mutation in DNA adeninemethylase gene and defective in macrophage intracellular survival (mutation in two component regulatory gene)	SV4089	*Eimeria tenella* (coccidiosis)	(Du & Wang 2005)
	Δcya, Δcrp	Lacks the adenylate cyclise and cyclic AMP receptor proteins	χ^{4064}	*Brucella abortus* (brucellosis)	(Stabel *et al.* 1991)
	phoP	Defective in macrophage intracellular survival (mutation in two component regulatory gene)	phoP^{c}	*Helicobacter pylori* (gastritis and gastric ulcers)	(Corthesy-Theulaz *et al.* 1998)
	gale	Inactivation that leads to galactose-induced cell lysis, interfering with Vi polysaccharide production	Ty21a	*E. coli* (diarrhea)	(Clements & El-Morshidy 1984)
Salmonella enterica serovar *typhi*	*aroA, aroC*	Double *aro* mutant	Ty2	*Clostridium tetani* (tetanus)	(Chatfield *et al.* 1992)
	ΔguaBA	Deletion mutation that impairs guanine nucleotide biosynthesis	CVD 915	*Clostridium tetani* (tetanus)	(Chatfield *et al.* 1992)
	aroA his	Impaired in their ability to produce aromatic amino acids required for in vivo replication, histamine requiring mutant	SL1438	*E. coli* (diarrhea)	(Bost & Clements 1995)

Salmonella enterica serovar *typhimurium* and *Salmonella enterica* serovar *typhi* can evade the host immune immune system. The mechanism, by which this is accomplished, was thought to involve the induction of phagocytosis by DC's. Recently Tobar *et al.* (2006) described this mechanism in more detail, explaining that these bacteria prevent degradation by lysosomes, allowing proliferation. Mutated *Salmonella* spp. are attenuated in their ability to evade the host system by this mechanism as shown in figure 2.8 (Bost & Clements 1995). Once the bacterium is phagocytosed by an APC, the lysosome degrades the bacterial carrier (Tobar *et al.* 2006) releasing the DNA plasmid vaccine into the cytosol. The DNA plasmid then moves to the nucleus for antigen gene expression. From here, the adaptive immune response can be elicited, the first method is by direct priming, the antigen released in the cytosol is processed by the APC and presented to T-cytotoxic cells via MHC class I. The second method involves cross-priming, where the processed antigen is released, after apoptosis, from the cell and internalized by another APC. The antigen is then processed in an endosome and presented via MHC class II molecules to T-helper cells. Lastly microbe-associated molecular patterns (MAPS) can be recognised by Toll-like receptors (TLR) to elicit an innate and adaptive immune response (Xu & Ulmer 2003).

Salmonella spp. have a number of MAPS recognized by the host. Among the most important are lipopolysaccharides (LPS) and flagellin. LPS, found on the outer membrane of the bacterium, is recognized by Toll-like receptor 4 (TLR4) to stimulate the innate immune system. Flagellin, recognised by TLR5, forms part of the filaments that make up the flagellum of the bacteria and translocate across the epithelial barrier inducing an inflammatory response (Pan *et al.* 2009). Once the bacteria is internalised by DCs the bacterium is processed and the peptides presented on the cell surface. Helper T cells then recognise antigen bound MHC class II molecules and are activated to provide help for immunoglobulin A (IgA) committed B cell development (figure 2.8). This is followed by affinity maturation to increase the affinity of the antibody for the antigen. The $CD4^+$ T cells and IgA^+ B cells migrate from the inductive sites, via the efferent lymphatic vessels, to the mesenteric lymph nodes. From the lymph nodes, the cells migrate, via the thoracic duct and blood circulation to the effector sites. The effector sites include the glandular tissues and lamina propria of both the respiratory and intestinal tracts. $CD4^+$ T helper cell type 2 (T_h2) cells produce various cytokines, such as interleukin-5 (IL-5) and IL-6, which stimulate the differentiation of the immature IgA^+ B cells into mature IgA producing plasma cells. The plasma cells produce IgA, in the dimeric or polymeric forms, which bind to Ig receptors. The secretory IgA are released from the monolayer of the epithelial cells into the nasal passage and intestinal tract, respectively (Kiyono, Fukuyama 2004).

If all of the factors discussed before are taken into account, the *Salmonella enterica* serovar *typhimurium* SL3261 bacterial strain thus holds excellent potential as a DNA vaccine carrier for use in ostriches which was the strategy followed in this study. The preparation and stability of mucosal vaccines against the ostrich mycoplasma, *Mycoplasma struthionis*, using *Salmonella enterica* serovar *typhimurium* SL3261 as a carrier bacteria will be described and the results of the vaccination trials with these vaccines will presented in the chapters that follow.

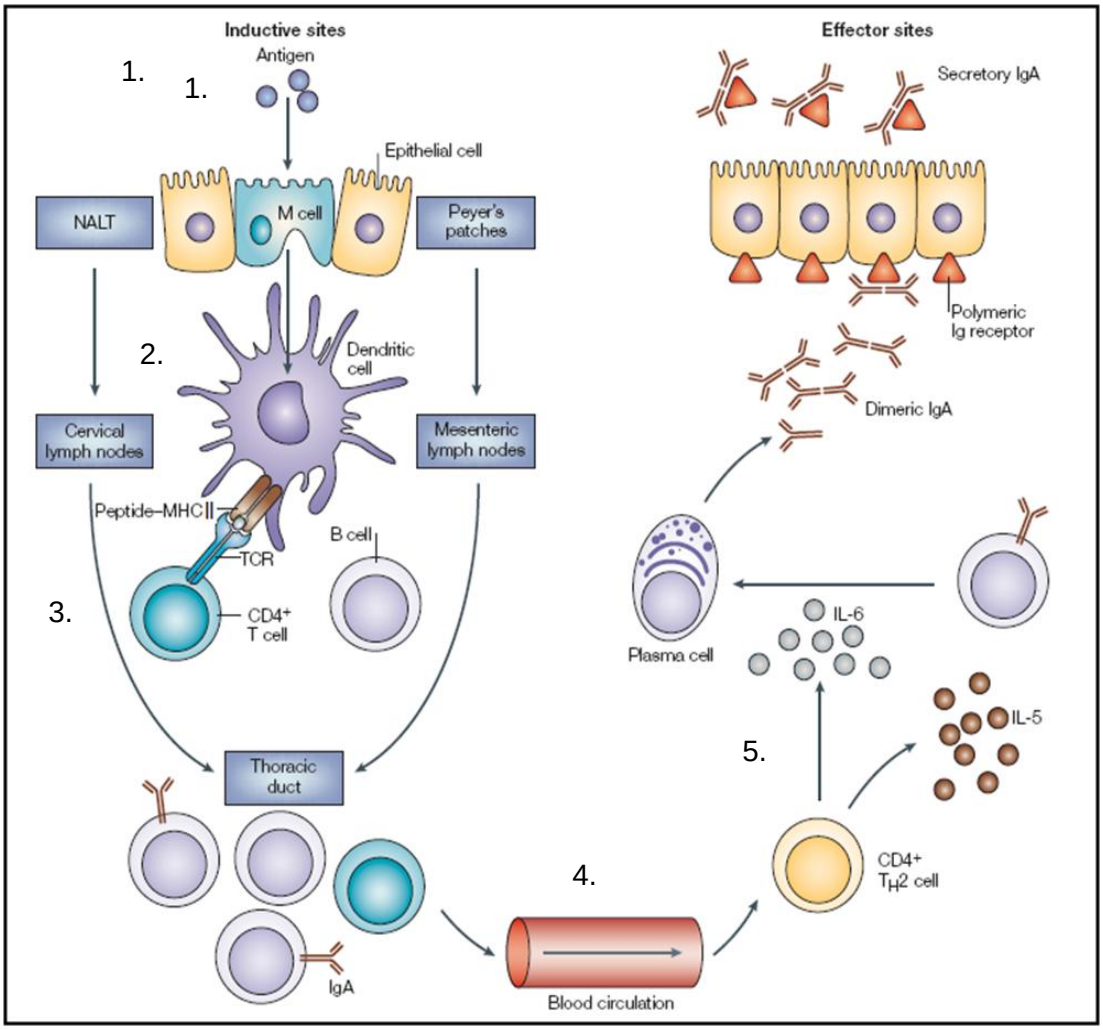

Figure 2.7: The mucosal immune response (Kiyono & Fukuyama 2004): 1. Antigens, introduced intranasally or orally, invade the M cells that line the epithelial layer. 2. APC internalize the bacteria, process the bacterial antigens and present the antigens on their surfaces, bound to MHC class II molecules. 3. Helper T cells recognise antigen bound MHC class II molecules and are activated to provide help for IgA committed B cell development. 4. The $CD4^+$ T cells and IgA^+ B cells migrate from the inductive sites, via the efferent lymphatic vessels, to the cervical lymph nodes and to the mesenteric lymph nodes for NALT and Peyer's patches respectively. From the respective lymph nodes, the cells migrate, via the thoracic duct and blood circulation to the effector sites. 5. $CD4^+$ T helper cell type 2 (T_h2) cells produce various cytokines, such as interleukin-5 (IL-5) and IL-6, which stimulates the differentiation of the immature IgA^+ B cells into mature IgA producing plasma cells. The plasma cells produce IgA, in the dimeric or polymeric forms, which bind to Ig receptors. Secretory IgA is released from the monolayer of the epithelial cells into the nasal passage and intestinal tract, respectively.

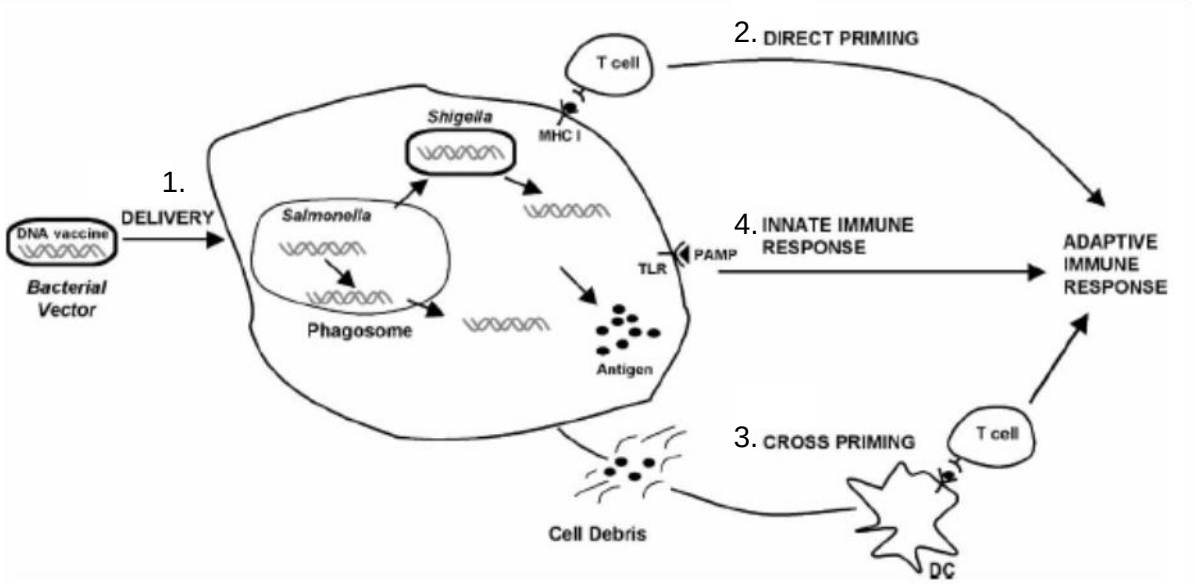

Figure 2.8: Delivery of DNA plasmid vaccine by attenuated *Salmonella* spp. taken from Xu & Ulmer (2003). 1. The bacteria are internalized by an APC where lysosomes degrade the bacterial carrier releasing the DNA plasmid vaccine into the cytosol. The DNA plasmid moves to the nucleus for expression of antigen. The immune response can be primed by *Salmonella* spp. in various ways. 2. The first method is by direct priming. The antigen is processed by the APC and presented to T cells via MHC class I molecules on the surface of the APC. 3. An adaptive immune response can also be elicited by cross priming. The processed antigen is released, after apoptosis, from the cell and internalized by another APC. The antigen is then processed and presented via MHC class I molecules to T cells. 4. Lastly MAPS can be recognised by Toll-like receptors (TLR) to elicit an innate and adaptive immune response.

Chapter 3: Oral vaccine preparation

3.1. Introduction

The effectiveness of a DNA vaccine depends mainly on the immunogenicity of the chosen antigen, and the level of gene expression in eukaryotic cells (Raupach & Kaufmann 2001). The elements of the bacterial plasmid into which the vaccine candidate gene is inserted also plays a role in the ability of the DNA vaccine to elicit an immune response. The most important factors include an ori, which directs the production of high plasmid yields, a strong promoter and polyA termination sequence that all influence the level of gene expression and directs expression of the candidate gene and an antibiotic resistance gene for successful transformation selection (Alpar, Papanicolaou & Bramwell 2005, Dufour 2001). Other elements that can be included are an intron following the promoter for higher expression levels, a signal sequence, such as tPA, to direct secretion of the translated protein, cytidine-phosphate-guanosine (CpG) motifs can also be included to increase the antigen-specific immune response (Donnelly *et al.* 1997). The routes of DNA vaccine delivery include mostly intramuscularly, but to produce a mucosal immune response the oral or intranasal routes are suggested (Ertl & Thomsen 2003). The vaccine delivery systems can also play a role in the ability and effectiveness of a DNA vaccine to elicit an immune response. Routes that have been investigated to protect the DNA vaccine from extracellular degradation are encapsulated in biodegradable polyester particles, enclosed in liposomes and delivery via bacterial carriers (Gurunathan, Klinman & Seder 2000). Other factors that also play a role include the frequency of immunization and the localisation of the expressed antigen (Alpar, Papanicolaou & Bramwell 2005, Gurunathan, Klinman & Seder 2000). The choice of each of these factors differs from one DNA vaccine to the next and various strategies can be investigated to ensure that the DNA vaccine is capable of eliciting an immune response and confer protection.

Pretorius (2009) identified the vaccine candidate gene, *oppA* in *M. struthionis* using bioinformatic analysis, which forms part of the oligopeptide permease system. The gene was isolated and transformed into each of three plasmids; pCI-neo, VR1012 and VR1020. Brandt (2012) injected the ostriches intramuscularly with each of the three naked DNA vaccines, but the results were inconclusive due to an outbreak of avian influenza (AI) that influenced the outcome of the vaccine trial. In the attempt to create an effective ostrich-specific mycoplasma vaccine the use of a bacterial carrier to deliver the DNA vaccine was considered as a potential improvement, but more importantly because a mucosal immune response was actually required as *Mycoplasma struthionis* infects the respiratory tract of ostriches. *Salmonella enterica* serovar *typhimurium* SL3261 was chosen as a potential carrier because it contains a deletion in the *aroA* gene limiting its growth *in vivo*. This strain is also capable of eliciting a humoral and mucosal immune response (Dufour 2001) and *Salmonella* species are common pathogens of the ostrich gastro-intestinal tract.

To prepare the mucosal DNA vaccine each of the three naked DNA vaccines had to be prepared in sufficient amounts. To this end the vaccines were first transformed in *E. coli* cells for proliferation, and then purified. To insert a DNA vaccine into *Salmonella enterica* serovar *typhimurium* SL3261 the method of electroporation is often used. Electroporation (figure 3.1) is a method by which an electrical field is applied to transform the bacterial cell with the plasmid (Sanderson, MacLachlan, & Hessel 1995). Electroporation is preferentially used because it is a faster transformation method, with a higher efficiency than chemical methods (O'Callaghan & Charbit 1990, Taketo 1988). For this reason each of the three DNA vaccines were electroporated into the carrier bacteria and the *in vivo* stability of the DNA vaccines in the bacterial carrier was also tested.

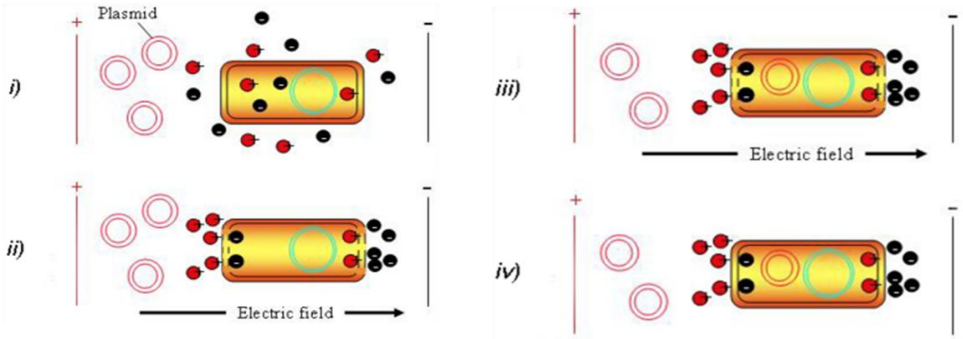

Figure 3.1: Electroporation i. Competent bacterial cells and DNA plasmids are mixed in a cuvette. ii. An electrical field is applied and the ions move according to charge. iii. Pathways form across the bacterial cell membrane which allows the entry of plasmid DNA into the cell. iv. When the electrical field is removed, the cell membrane repairs itself, closing the pathways.

3.2. Materials and Methods

3.2.1. Naked DNA vaccines

3.2.1.1. Transformation

The recombinant plasmids pCI-neo, VR1012 and VR1020, containing the *oppA* gene, were previously prepared in the laboratory (Pretorius, 2009). An amount of 50 ng of each vector was mixed with 50 µl competent JM109 *E. coli* cells in a 17 x 100 mm polypropylene round-bottom tube (14 ml Falcon® tube) and incubated on ice for 10 min. The mixture was heat shocked in a 42°C water bath for 45 sec and then incubated on ice for 2 min. The transformed cells were incubated in 950 µl of Luria broth (LB) medium (10 g/L Bacto-tryptone (Biolab, Merck), 5 g/L Bacto-yeast extract (Biolab, Merck), 5 g/L NaCl and pH set to 7) at 37°C, and rotated on an orbital shaker (IKA® K260 basic) at 150 rpm for 30 min. One hundred microliter of the transformed cells were plated onto plastic Petri dishes containing LB agar (LB medium with 15 g/L agar (Biolab, Merck). For the recombinant plasmid pCI-neo, the antibiotic ampicillin (100 µg/ml, Sigma-Aldrich) was added to the

LB agar plates and for the recombinant plasmids VR1012 and VR1020 the antibiotic kanamycin (100 µg/ml, Biochemica, Fluka). The plates were incubated at 37°C for 16 hours.

The successful transformation of the recombinant plasmids into the JM109 *E. coli* cells was tested using a diagnostic colony PCR. The primer pairs used to test for the positive transformants are shown in Table 3.1. The PCR reaction mixture contained 1 µl of a 10x reaction buffer, 0.6 µl of a 25 mM $MgCl_2$, 0.5 µl of each primer (20 pmol/µl), 0.4 µl deoxyribonucleotide triphosphate (dNTP) mix (containing 5 mM of each dATP, dCTP, dGTP and dTTP) (Bioline), 0.025 U/µl Taq DNA polymerase (Supertherm polymerase, JMR Holdings), and 6.9 µl autoclaved Milli-Q water. A colony was picked from the LB agar plates using a sterilized toothpick and mixed in the PCR mixture to release some of the bacteria. The PCR was conducted under the following conditions: the first denaturation cycle at 95°C for 5 min; then 25 cycles each consisting of a 30 sec denaturation step at 94°C, a 30 sec annealing step at 55°C and a 30 sec extension step at 72°C; followed by a final extension cycle at 72°C for 7 min. This PCR reaction was carried out in a Veriti 96-well thermal cycler from Applied Biosystems.

Table 3.1: Primer sets chosen for each of the recombinant vectors

Plasmid	Primer	Sequence	Estimated size
pCI-neo	T7(EEV)	5'-AAGGCTAGAGTACTTAATACGA-3'	3000 bp
	T3	5'-AATTAACCCTCACTAAAGGG-3'	
VR1012	VR1012_F	5'-CGCGCCACCAGACATAATAG-3'	900 bp
	P100_seqP 1R	5'-CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG-3'	
VR1020	VR1020_seq_F	5'-CGTCGACAGAGCTGAGATCCTACAG-3'	900 bp
	P100_seqP 1R	5'-CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG-3'	

The PCR products were analysed by gel electrophoresis on a 1% (w/v) agarose gel (Seakem® LE agarose for gel electrophoresis, Lonza) in 1x TAE buffer (40 mM Tris-acetate; 20 mM glacial acetic acid and 1 mM EDTA, pH 8.0) and 0.175 µg/ml ethidium bromide for DNA visualization. The PCR products were prepared for loading onto the gel by mixing 10 µl of PCR product with 0.1 volumes of gel loading buffer (50% (v/v) glycerol; 0.1% (v/v) bromophenol blue; 50 mM EDTA and 100 mM Tris-base, pH 8.0). The samples were then electrophoresed (100 V, ~60 min) and the DNA visualized under ultraviolet (UV) light.

3.2.1.2. Plasmid purification

For each of the three DNA vaccines four fresh starter cultures were prepared in 14 ml Falcon® tubes by inoculating 5 ml LB medium containing antibiotic with a single positively transformed colony from the LB agar plates produced in section 3.2.1.1. The tubes were incubated for 8 hours at 37°C, shaking at 200 rpm on an orbital shaker (IKA® K260 basic). Two large overnight cultures

were prepared by inoculating 100 ml LB medium, containing antibiotic, with 2 ml of the 5 ml starter culture, prepared above, and incubating at 37°C, shaking on an orbital shaker at 200 rpm for 16 hours. After 16 hours the OD_{600} of each overnight culture was measured and the recommended culture volume to be used in the Nucleobond® Xtra Midi Endotoxin-free plasmid purification kit (Macherey-Nagel) was calculated using the following equation:

$$V[mL] = 400 / OD_{600}.$$

The cells from the overnight cultures were harvested by centrifugation at 6,000 x *g* for 10 min at 4°C. The pellet was resuspended in 8 ml resuspension buffer (RES-EF) containing RNase A. Eight millilitres of Lysis buffer (LYS-EF) was added and the tube inverted five times. While the tube was incubated at room temperature for 5 min, 15 ml of equilibration buffer (EQU-EF) was used to equilibrate the Nucleobond® Xtra Column filter. After the 5 min incubation period, 8 ml of neutralization buffer (NEU-EF) was added and the tube inverted 10-15 times. For each of the following steps the Nucleobond® Xtra Column was allowed to empty by gravity flow. After the crude cell lysate was incubated on ice for 5 min, the tube was inverted 3 more times to obtain a homogenous solution and was then loaded onto the column. Before the column filter was removed, 5 ml of wash buffer (FIL-EF) was added to wash the residual lysate onto the column. The column was then washed two more times to remove any endotoxins. The second wash was with 35 ml wash buffer (ENDO-EF) and the third wash was with 15 ml wash buffer (WASH-EF). Once the column was properly washed and allowed to empty by gravity flow the DNA was eluted with 5 ml elution buffer (ELU-EF) and collected in a 14 ml Falcon® tube. A Nucleobond® finalizer was used to concentrate and desalinate the DNA vaccine. The salt in the eluate was precipitated by 0.7 volumes of room temperature isopropanol incubated at room temperature for 2 min. The mixture was then added to a 30 ml syringe, with the Nucleobond® Finalizer attached, and slowly pressed through with a constant force. For every following step, the Nucleobond® Finalizer was removed from the syringe before the plunger was removed and the Nucleobond® Finalizer was then reattached. The flow-through was discarded and 2 ml endotoxin-free 70% ethanol was added and slowly pressed through the Nucleobond® Finalizer. The Nucleobond® Finalizer was then cleared of the ethanol by pushing air through until it was dry. The Nucleobond® Finalizer was transferred to a 1 ml syringe and the purified plasmid eluted into an 1.5 ml Eppendorf tube with 500 µl endotoxin-free H_2O. The concentration of the purified DNA vaccines were measured on a nanodrop ND 1000 spectrophotometer and the purified DNA vaccines were stored at 4°C until use. The purified DNA vaccine was also analysed by gel electrophoresis on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate; 20 mM glacial acetic acid and 1 mM EDTA, pH 8.0) and 0.175 µg/ml ethidium bromide as the DNA stain. The plasmids were prepared for loading onto the gel by mixing 2 µl of plasmid with 0.1 volumes of gel loading buffer (50% (v/v) glycerol; 0.1% (v/v) bromophenol blue; 50 mM EDTA and 100 mM Tris-base, pH 8.0). The samples were then electrophoresed (100 V, ~60 min) and the DNA visualized under ultraviolet (UV) light.

3.2.2. Mucosal DNA vaccine preparation

3.2.2.1. Salmonella enterica *serovar* typhimurium *SL3261*

Salmonella enterica serovar *typhimurium* SL3261 was received from the *Salmonella* Genetic Stock Centre (SGSC) (Dr. K. E. Sanderson from the University of Calgary). On arrival the strain was immediately streaked on plastic Petri dishes containing Tryptic soy agar (TSA) (Biolab, Merck) and incubated at 37°C for 24 hours. One or two colonies was selected from the TSA plate and streaked on Xylose lysine deoxycholate (XLD) agar (Biolab, Merck) and incubated at 37°C for 24 hours. A few of the *Salmonella* colonies were incubated in 5 ml Tryptic soy broth (TSB) (Biolab, Merck) and incubated at 37°C for 24 hours, shaking at 200 rpm on an orbital shaker. *Salmonella enterica* serovar *typhimurium* SL3261 freezer stocks were prepared from the TSB cultures by mixing with an equal volume of 30% (v/v) glycerol and stored at -80°C for up to one year.

3.2.2.2. Electroporation of DNA vaccines into Salmonella enterica *serovar* typhimurium *SL3261*

Each of the DNA vaccines; were transformed into *Salmonella enterica* serovar *typhimurium* SL3261 by electroporation adapting the method published by Sanderson, MacLachlan & Hessel (1995). Electroporation competent *Salmonella enterica* serovar *typhimurium* SL3261 cells were prepared as follows. A fresh overnight culture was prepared in a 14 ml Falcon® by inoculating 5 ml TSB with *Salmonella enterica* serovar *typhimurium* SL3261 freezer stocks prepared in section 3.2.2.1, using a loop. The fresh overnight culture was incubated at 37°C shaking at 200 rpm on an orbital shaker for 16-18 hours. Two 100 ml TSB cultures were each inoculated with 1 ml of the fresh overnight culture and grown at 37°C with shaking at 200 rpm on an orbital shaker to an OD_{600} of 0.75, as measured with a DU 650 spectrophotometer (Beckman). The cells were chilled on ice for 15 min and then centrifuged at 4,000 x *g,* 4°C for 10 min in an Avanti J-E centrifuge (Beckman). The supernatant was discarded and the pellet resuspended in cold distilled water equal to the original culture volume and centrifuged as before. The supernatant was discarded again and the pellet resuspended in a half of the original culture volume of cold distilled water and centrifuged again as before. The pellet was resuspended in 1/50 the original culture volume of 10% (v/v) glycerol and centrifuged at 4,000 x *g,* 4°C for 10 min. The pellet was resuspended in 1/100 the original culture volume of 10% (v/v) glycerol. The electrocompetent cells were kept on ice until use. If the cells were not used immediately for electroporation, they were stored at -80°C until use.

Electrotransformation was done as follows. In an 1.5 ml Eppendorf tube, 40 µl of the competent *Salmonella enterica* serovar *typhimurium* SL3261 cells were mixed with 50 ng DNA vaccine in 2 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Using a Pasteur pipette the entire contents of the Eppendorf tube was transferred to a chilled electroporation cuvette (Gene pulser cuvette with a 0.2 cm electrode gap, Bio-rad) and all the moisture removed from the outside. The mixture was pulsed once in a Gene pulser II (Bio-Rad) set at 12.5 kV/cm, 25 µF capacitance and 200 Ω resistance to

yield a pulse length of 5 m/s. The mixture was added to 1 ml Super optimal broth (SOC) medium (20 g/L Bacto-tryptone (Biolab, Merck), 5 g/L Bacto-yeast extract (Biolab, Merck), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) in a 14 ml Falcon® tube and incubated at 37°C for 1 hour without shaking. One hundred microliter of the transformed cell suspension was plated onto plastic Petri dishes containing TSA with antibiotic; for the DNA vaccine pCI-neo 100 µg/ml ampicllin was used and for the DNA vaccines VR1012 and VR1020 100 µg/ml kanamycin was used. The plates were incubated at 37°C for 16-18 hours.

The successful electrotransformation of the recombinant vectors into *Salmonella enterica* serovar *typhimurium* SL3261 cells were tested using the diagnostic colony PCR described in section 3.2.1.1. The primers used for each recombinant vector are listed in Table 3.2. Following amplification the PCR products were analysed on a 2% (w/v) agarose gel (Seakem® LE agarose for gel electrophoresis, Lonza) in 1x TAE buffer (40 mM Tris-acetate; 20 mM glacial acetic acid and 1 mM EDTA, pH 8.0) and 0.175 µg/ml ethidium bromide as the DNA stain. The agarose gel was visualized under UV light. To prepare freezer stocks of the successful transformants, 2 positive colonies selected, from the TSA plates, were added to a 14 ml Falcon® tube containing 5 ml of TSB containing antibiotic, ampicillin (100 µg/ml, Sigma-Aldrich) for the pCI-neo transformants and kanamycin (100 µg/ml, Biochemica, Fluka) for the VR1012 and VR1020 transformants. The Falcon® tubes were incubated at 37°C shaking at 200 rpm on an orbital shaker for 16-18 hours. The TSB cultures were then mixed with an equal volume of 30% (v/v) glycerol and stored at -80°C for up to one year.

Table 3.2: Primer sets for each of the recombinant vectors

Plasmid	Primer	Sequence	Size (bp)
pCI-neo	T7(EEV)	5'-AAGGCTAGAGTACTTAATACGA-3'	3000
	T3	5'-AATTAACCCTCACTAAAGGG-3'	
VR1012	VR1012_F	5'-CGCGCCACCAGACATAATAG-3'	1500
	P100_seqP2R	5'-CAATTGTTTGATTTGTTCTTAGCCATGAATAATAGAAATC TTTAG-3'	
VR1020	VR1020_seq_F	5'-CGTCGACAGAGCTGAGATCCTACAG-3'	1500
	P100_seqP2R	5'-CAATTGTTTGATTTGTTCTTAGCCATGAATAATAGAAATC TTTAG-3'	

3.2.2.3. Stability of DNA vaccines in Salmonella enterica *serovar* typhimurium *SL3261*

To test persistence of the DNA vaccine after transformation into *Salmonella enterica* serovar *typhimurium* SL3261, the following method was followed. A fresh overnight culture of each of the three mucosal DNA vaccines was prepared by inoculating 5 ml TSB containing antibiotic, 100 µg/ml ampicllin for the pCI-neo DNA vaccine and 100 µg/ml kanamycin for the VR1012 and VR1020. The cultures were incubated shaking at 200 rpm on an orbital shaker at 37°C for 18

hours. For each of the three mucosal DNA vaccine constructs two Petri dishes containing TSA and antibiotic and two Petri dishes containing only TSA was streaked with the fresh overnight culture. The plates were incubated at 37°C for 18 hours. The diagnostic colony PCR described in section 3.2.1.1. and the primer sets in Table 3.2 were used to test 10 randomly selected colonies on the TSA plates with antibiotic and 10 randomly selected colonies from the plates without antibiotic. The PCR products were analysed on a 1% agarose gel prepared as in section 3.2.1.1. and visualized under UV light. One positive colony from each TSA plate was selected and streaked onto a new set of TSA plates, the plates were then incubated at 37°C for 18 hours. The diagnostic colony PCR described in section 3.2.1.1. and the primer sets in Table 3.1 were once again used to test 10 colonies on the TSA plates with antibiotic and 10 colonies from the plates without antibiotic. This was repeated for 100 generations (5 days).

3.3. Results

3.3.1. Naked DNA vaccines

3.3.1.1. Transformation

JM109 *E. coli* cells were successfully transformed with the DNA vaccines containing the *oppA* gene using heat shock. Transformation was confirmed by testing randomly selected colonies from the antibiotic containing LB agar plates and using a colony PCR and the primer sets in Table 3.1 (figure 3.2 and 3.3). The colony PCR used to test for the successful transformation with recombinant pCI-neo plasmid produced an amplification product of 3000 bp. The colony PCR product to test for the successful transformation with the recombinant VR1012 or VR1020 plasmids produced amplification products of 900 bp.

3.3.1.2. *Plasmid purification*

The Nucleobond® Xtra Midi Endotoxin-free plasmid purification kit was used to successfully purify the transforments as can be seen in figure 3.4. The bands in lane 2 and 3 (approximately 8000 bp) represents the isolated pCI-neo DNA vaccine expected to be 8301 bp. The difference in size is due to a supercoiled structure formation of the pCI-neo plasmid. The bands in lanes 4 to 5 and 6 to 7 (between 6000 and 8000 bp) represents the isolated VR1020 and VR1012 DNA vaccines, respectively. These bands are found in the expected size ranges of the VR1020 (7873 bp) and VR1012 (7740 bp) DNA vaccines.

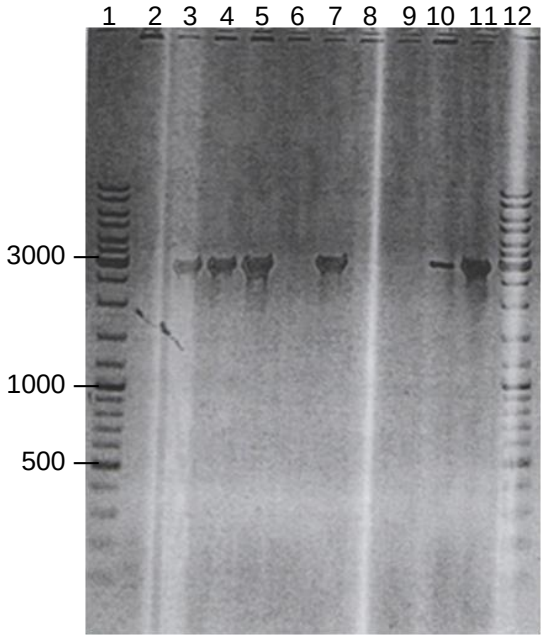

Figure 3.2: Transformation products of transformation of JM109 *E. coli* cells with the recombinant pCI-neo plasmid shown on a 1% (w/v) agarose gel. Lane 1 and 12: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 2-11: 10 µl diagnostic colony PCR product using randomly selected colonies from LB agar plates.

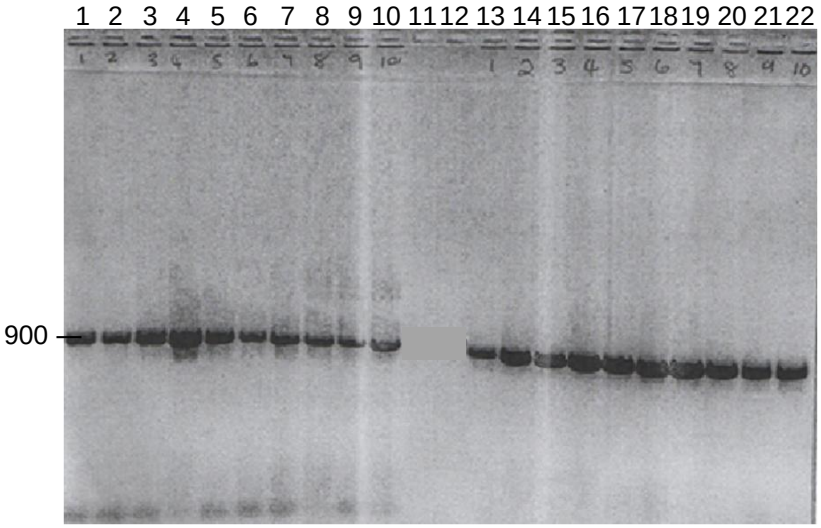

Figure 3.3: Transformation products of transformation of JM109 *E. coli* cells with the recombinant plasmids VR1012 and VR1020 shown on a 1% (w/v) agarose gel. Lane 1-10: 10 µl diagnostic colony PCR product using randomly selected colonies from LB agar plates with recombinant VR1012 vaccine transformants. Lane 13-22: 10 µl diagnostic colony PCR product using randomly selected colonies from LB agar plates with recombinant VR1020 vaccine transformants.

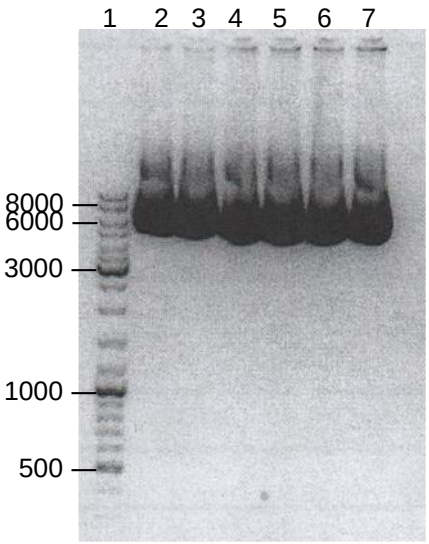

Figure 3.4: Purified plasmid viewed on a 1% (w/v) agarose gel. Lane 1: 5 µl DNA Ladder mix (Generuler™, Fermentas). Lane 2 and 3: Purified recombinant pCI-neo plasmid. Lane 4 and 5: Purified recombinant VR1020 plasmid. Lane 6 and 7: Purified recombinant VR1012 plasmid.

3.3.2. Mucosal DNA vaccine preparation

3.3.2.1. Salmonella enterica *serovar* typhimurium *SL3261*

XLD medium is a selective growth medium used to isolate *Salmonella* and *Shigella* species. Wild type *Salmonella* produce hydrogen sulphide (H_2S) resulting in red colonies with a black center. *Salmonella enterica* serovar *typhimurium* SL3261 is an *aroA* mutant and does not produce H_2S, resulting in translucent colonies on the XLD agar plates (figure 3.5) (O'Callaghan, Charbit 1990, Taketo 1988).

i. ii.

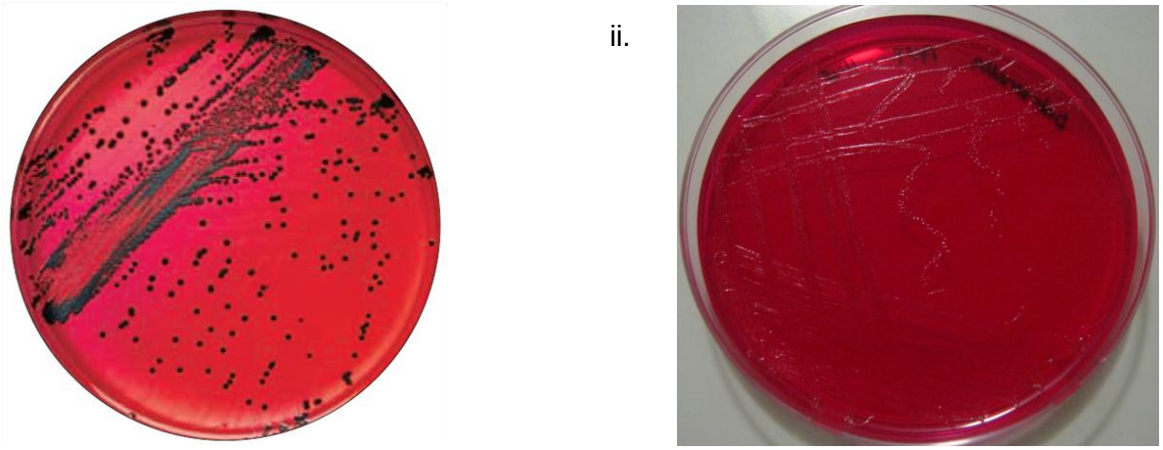

Figure 3.5: XLD medium: i. Wild type *Salmonella* growth displayed as red colonies with a black center taken from (Zimbro *et al.* 2009). ii. Mutant strain *Salmonella enterica* serovar *typhimurium* SL3261 displayed as translucent colonies photo taken by Amanda van Tonder.

3.3.2.2. *Electroporation of DNA vaccines into* Salmonella enterica *serovar* typhimurium *SL3261*

The electrotransformation of *Salmonella enterica* serovar *typhimurium* SL3261 with each of the DNA vaccines was carried out successfully by adapting the method of Sanderson, MacLachlan & Hessel (1995). Figure 3.6 and 3.7 show the products of a diagnostic colony PCR where primer sets in Table 3.2 were used to test the successful electroporation. The approximate product size expected for successful electroporation of the pCI-neo DNA vaccine was 3000 bp and for the VR1012 and VR1020 DNA vaccines was 1500 bp. The electroporation of the recombinant VR1012 and VR1020 vaccines was more successful than that of the recombinant pCI-neo vaccine.

3.3.2.3. Stability of DNA vaccines in Salmonella enterica *serovar* typhimurium *SL3261*

The stability of the DNA vaccines in *Salmonella enterica* serovar *typhimurium* SL3261 was tested for 100 generations in the absence and presence of antibiotic, to assess the effect of an environment free of antibiotics and the ability of the carrier bacteria to retain the plasmid, respectively. After the first day all the colonies tested were positive. From the second day the number of selected colonies testing positive for the pCI-neo recombinant vaccine fluctuated both in the presence and absence of antibiotic. The number of chosen colonies that tested positive for the VR1012 DNA vaccines remained relatively the same, with some colonies testing negative on the plates grown without antibiotic, especially on the fifth day (figure 3.8). For the VR1020 DNA vaccine, the results observed were very similar to those obtained with the VR1012 DNA vaccine.

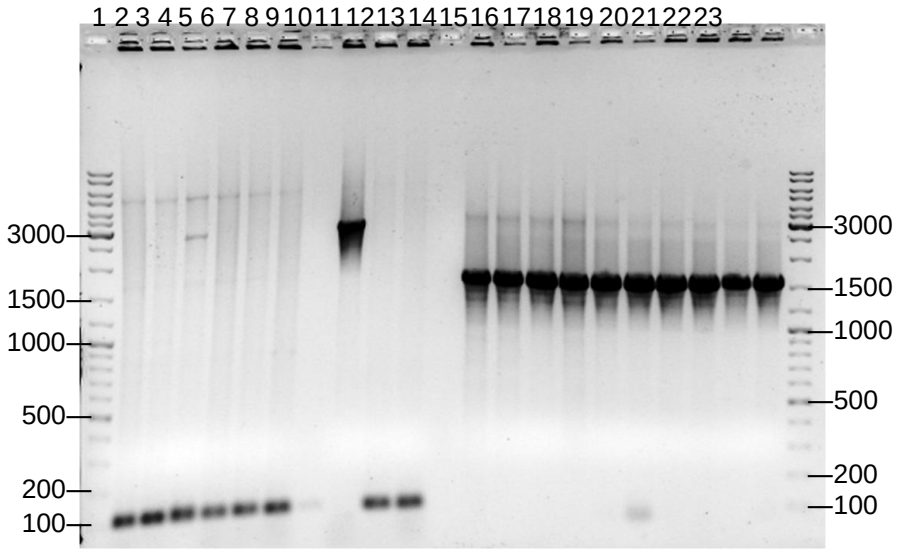

Figure 3.6: Diagnostic colony PCR of electroporation products. Lane 1: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 2-11: 10 µl PCR product from selected colonies on LB agar plates with transformants from electroporation of *Salmonella enterica* serovar *typhimurium* SL3261 with recombinant pCI-neo plasmid containing *oppA* gene. Lane 13-22: 10 µl PCR product from selected colonies on LB agar plates with transformants from electroporation of *Salmonella enterica* serovar *typhimurium* SL3261 with recombinant VR1012 plasmid containing *oppA* gene. Lane 23: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas)

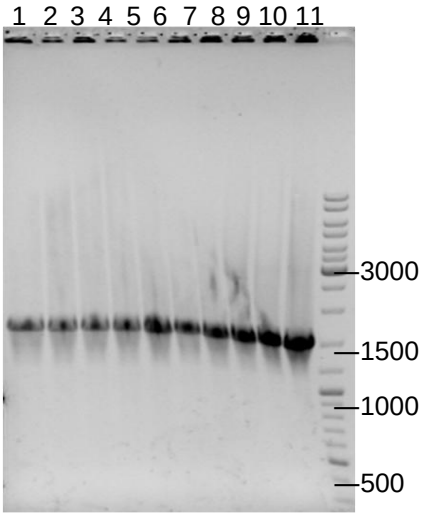

Figure 3.7: Diagnostic colony PCR of electroporation products. Lane 1-10: 10 µl PCR product from selected colonies on LB agar plates with transformants from electroporation of *S. enterica* serovar *Salmonella enterica* serovar *typhimurium* SL3261 with recombinant VR1012 plasmid containing *oppA* gene. Lane 11: 5 µl DNA Ladder mix (Generuler™, Fermentas).

3.4. Discussion

Each of the recombinant plasmids, pCI-neo, VR1012 and VR1020, containing the *oppA* gene, was successfully transformed into JM109 *E. coli* cells for plasmid amplification. The plasmid was purified using a Nucleobond® Xtra Midi Endotoxin-free plasmid purification kit. The purified recombinant plasmid was used previously as a naked DNA vaccine and its efficacy tested in a preliminary vaccine trial as an intramuscular vaccine, but the trial was influenced by an outbreak of AI giving inconclusive results (Brandt 2012). As *Mycoplasma struthionis* causes infection in the respiratory tract of ostriches, a mucosal immune response is required.

A literature study revealed that the use of bacteria as carriers of DNA vaccine can stimulate both a mucosal and serum immune response. The use of *Salmonella* as bacterial vaccine carriers have shown promise in cats (Titball *et al.* 1997), dogs (Chabalgoity *et al.* 2000), pigs (Fagan *et al.* 1997, Stabel *et al.* 1993, Fagan *et al.* 2001) and chickens (Pogonka *et al.* 2003). *Salmonella enterica* serovar *typhimurium* SL3261 was chosen as a carrier to deliver the recombinant DNA plasmid vaccines. This strain is a highly attenuated *aroA* mutant with limited growth *in vivo* (Chen *et al.* 2006b, Hoiseth, Stocker 1981). XLD agar is a selective medium that contains a number of components that aid in the isolation of *Salmonella* and *Shigella* spp. Of note is the sodium thiosulfate and ferric ammonium citrate that is added to this medium. These two substances allows for the detection of H_2S production by the formation of black colonies (Zimbro *et al.* 2009). Wild type *Salmonella* spp. produce H_2S, resulting in red colonies with black centers. *Salmonella aroA* mutants do not produce H_2S and appear as translucent colonies on the XLD agar (Cooper *et al.* 1994).

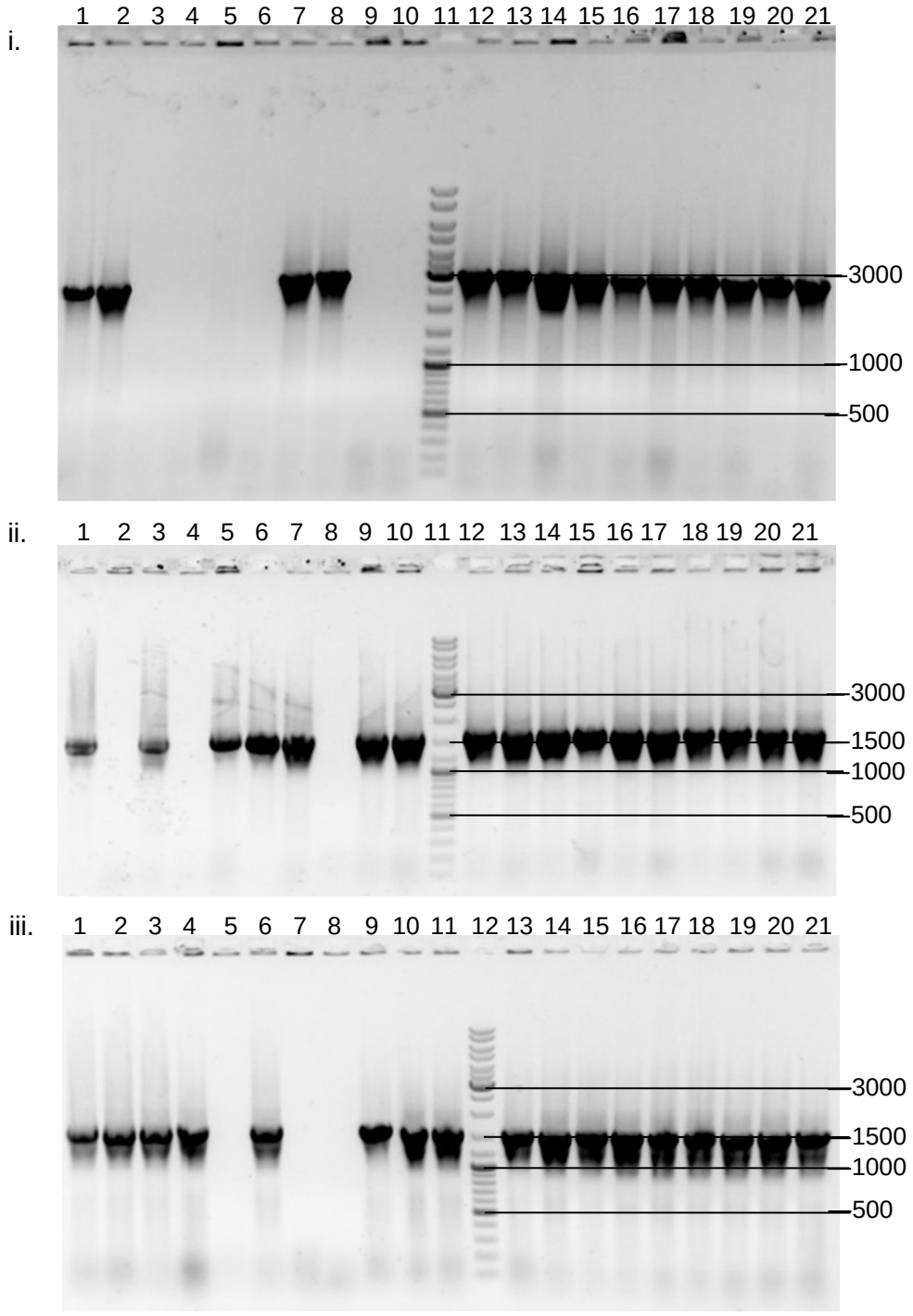

Figure 3.8: Day 5 of stability assessment of DNA vaccines in *Salmonella enterica* serovar *typhimurium* SL3261 tested with a diagnostic colony PCR. i. Mucosal pCI-neo DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, ampicillin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the the antibiotic ampicillin. ii. VR1012 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic kanamycin. iii. VR1020 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic kanamycin. Lane 12: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 11, 13-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic kanamycin.

The three recombinant plasmids were successfully transformed into *Salmonella enterica* serovar *typhimurium* SL3261 using electroporation to produce three mucosal DNA vaccines. The transformation efficiency for the recombinant plasmid pCI-neo was low, but for the recombinant plasmids VR1012 and VR1020 it was very high. The lower transformation efficiency of the pCI-neo DNA vaccine might be due to its size (8309 bp), which is much larger than that of the other DNA vaccines VR1012 (7740 bp) and VR1020 (7873 bp).

The stability of the DNA vaccines in the carrier bacterium *Salmonella enterica* serovar *typhimurium* SL3261 was tested for 100 generations with and without the selective pressure of antibiotic. Under selective pressure all three of the DNA vaccines, the carrier bacteria appear to be able retain the plasmids. In an antibiotic-free environment the VR1012 and VR1020 DNA vaccines were more stable than the recombinant plasmid pCI-neo, which showed variable stability. The higher tendency of *Salmonella enterica* serovar *typhimurium* SL3261 to retain the VR1012 and VR1020 DNA vaccines might also be due to the larger size of the pCI-neo DNA vaccine. Of the three mucosal DNA vaccines prepared, the VR1012 and VR1020 mucosal DNA vaccines appear to be more stable and might be a better choice for future vaccine trials. In the next chapter, the use of these mucosal DNA vaccines in a preliminary vaccine trial to test their ability to elicit immune responses is described.

Chapter 4: Preliminary vaccine trial

4.1. Introduction

An important step in vaccine development is to assess the vaccine's ability to elicit a specific acquired or adaptive immune response. Once this has been established, the ability of the vaccine to confer protection against the pathogen is evaluated by challenging the vaccinated animal with a virulent form of the pathogen indicating that the vaccine is able to prevent the disease caused by the pathogen. Together the effectiveness of these two processes is referred to as "vaccine efficacy". The specific acquired immune response elicited by a vaccine can be either a humoral or a cell-mediated immune response, and in some cases both types of immune responses can be elicited. During a humoral immune response B-cells produce antibodies in the serum and mucosa, and during a cell-mediated immune response T-helper or T-cytotoxic cells are activated and multiply (Cooper *et al.* 1994, Coombes & Powrie 2008). One major advantage of DNA vaccines is that they can induce both humoral and cell-mediated immune responses. However, the measurement of cell-mediated immune responses is complicated because this requires special tissue culture equipment, whilst the measurement of antibody levels is much easier (Wagner, Hewlett & Bloom 2009). The method most commonly used to test the humoral immune response induced by a vaccine is the enzyme-linked immunosorbent assay (ELISA). ELISAs are simple to perform, sensitive, fast and reliable systems that can be used to detect and quantify antibodies or antigens present in a sample (Wagner, Hewlett & Bloom 2009). Two separate research groups, Engvall and Perlmann from Sweden as well as Avrameas and Guilbert from the Netherlands, published the ELISA and enzyme immunoassay (EIA) techniques, respectively in 1971, as an alternative to the radioimmunoassay (RIA). ELISA is a versatile assay with many different types, such as competitive, direct, indirect and sandwich ELISA as well as combinations of these main types.

For the determination of the levels of antibodies elicited by the DNA vaccine, an indirect ELISA was used in this study. This method uses a labeled secondary antibody for the detection of the primary antibody present in a sample, making this method more sensitive (Hsieh 2010). This increase in sensitivity is the result of the binding of considerably more secondary antibodies than primary antibodies. The signal is further amplified by the use of the biotin/avidin detection system (Crowther 2000). Biotin is a molecule that can be coupled to an antibody, leaving the antigen binding capacity unaffected. Avidin is a tetrameric protein with four high affinity binding sites that binds with high specificity to biotin. Avidin can also be linked to enzyme. Such an indirect ELISA is shown in figure 4.1. The principle of this ELISA is that the amount of product formed, i.e. the final absorbance measured, is proportional to the amount of ostrich antibody present in the sample. In this chapter, this ELISA was used to assess the amounts of antibodies present in sera and saliva

to test the humoral and mucosal immune response against the different DNA vaccines that had been prepared.

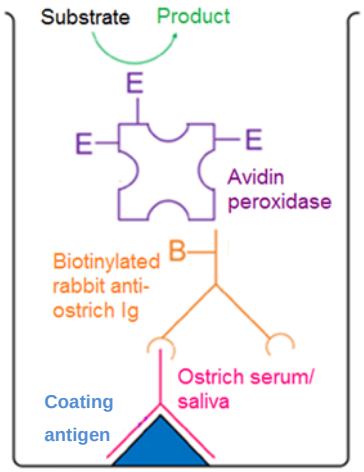

Figure 4.1: A diagram of the indirect ELISA used in this study. 1. The antigen is immobilised on a solid phase. 2. A sample to be tested for antibody presence is added and if antibodies specific for the antigen are present, they will bind. 3. A biotinylated secondary detector antibody is added that binds to the bound sample antibodies. 4. Peroxidase-labelled avidin is added which binds with high affinity and specificity to biotin. 5. The enzyme, peroxidase, acts as a catalyst when a substrate is added. The absorbance of the product that forms can then be measured

Pretorius (unpublished) generated three naked DNA vaccines in the laboratory against *Mycoplasma struthionis* using the method described in figure 2.5A. First a candidate gene, *oppA*, was identified and isolated, the gene was then cloned into each of three plasmid vectors, pCI-neo, VR1012 and VR1020 (Pretorius 2009). The naked DNA vaccines were prepared as described in Chapter 3 for electroporation into the carrier bacteria, *Salmonella enterica* serovar *typhimurium* SL3261, to generate three mucosal vaccines respectively. Of the three mucosal vaccines generated, the *Salmonella enterica* serovar *typhimurium* SL3261 carrying the VR1012 and VR1020 DNA vaccines were the most stable. One of the elements distinguishing the VR1020 plasmid from the VR1012 plasmid is the presence of a short signal sequence (tPA signal peptide) that signals a protein for the secretory pathway, which can possibly aid in the transfer of the membrane protein, OppA, to the outside of the cell. It is thought that the expression of higher levels of the vaccine protein extracellularly results in higher amounts of the protein being exposed to the antigen-specific B cells and thereby may lead to an improved immune response.yh Haddad *et al.* (1997) used COS cells to show that including a tPA signal in the vaccine plasmid results in the secretion of the translated protein through the endoplasmic reticulum and the Golgi apparatus. The tPA signal peptide has been used in vaccine plasmids and then used in both mucosal and naked DNA vaccines to elicit an immune response against the antigen of interest (Haddad *et al.* 1997, Parida *et al.* 2005).

In this chapter, the ability of the VR1020 mucosal DNA vaccine to elicit a humoral and mucosal immune response compared to the VR1020 naked DNA vaccine was evaluated. To this end, ostriches were immunized with the vaccines after being assessed for; possible prior *Salmonella* and ostrich *Mycoplasma* infections, and the ostriches' changes in weight were monitored. Six indirect ELISAs as illustrated above, were developed to analyse the humoral and mucosal immune responses of the ostriches against the *Mycoplasma struthionis* OppA protein, as well to the carrier bacteria, *Salmonella enterica* serovar *typhimurium* SL3261. To develop the ELISAs the OppA protein of *Mycoplasma struthionis* and LPS from *Salmonella enterica* serovar *typhimurium* SL3261 had to be isolated for use as coating agents. To assess the humoral immune response, secondary antibodies prepared against ostrich immunoglobulins (Ig) isolated from ostrich serum were previously prepared in this laboratory. To assess the mucosal immune response, secondary antibodies against ostrich IgA were required. Immunoglobulin A is the main antibody found in mucosal secretions. IgA found in the mucosal secretions is linked to a secretory piece, which aids in the transport of IgA across the mucosal epithelium into the mucosal slime layer, and protects it there against proteolysis. Botes (2004) isolated IgA from ostriches, ostrich IgA only, which is referred to as protein 1 here, and IgA to which the secretory piece is linked, which is referred to as protein 2 here. An immune response was elicited in rabbits against these isolated proteins to produce the secondary rabbit anti-ostrich IgA protein 1 and protein 2 (Botes 2004). Antibodies to protein 1 and protein 2 were both used as secondary antibodies in respective ELISAs to assess the mucosal immune responses of the ostriches in the preliminary vaccine trial. The results obtained were analysed statistically using an ANOVA to determine if the immune responses elicited by the naked DNA vaccine, injected intramuscularly, and the mucosal vaccine, administered orally, were statistically relevant.

4.2. Materials and Methods

4.2.1. Preliminary vaccine trial on a commercial ostrich farm in Oudtshoorn, Western Cape.

4.2.1.1. Vaccine trial design.

A vaccine trial to compare the VR1020 naked DNA vaccine and VR1020 mucosal DNA vaccine was performed on 120 ostriches on a commercial ostrich farm in Oudtshoorn, Western Cape, with ethical clearance from the University of Stellenbosch Animal Ethics Committee. The trial was designed so that there were 4 groups each consisting of 30 ostriches. One group received 100 µg/ml of the VR1020 naked DNA vaccine intramuscularly and one group received 10^9 c.f.u./ml of the VR1020 mucosal DNA vaccine orally. The last two groups were used as a control groups, one group received 10^9 c.f.u./ml of the *Salmonella enterica* serovar *typhimurium* SL3261 without the recombinant vaccine plasmid inserted and the other group received no vaccinations. The vaccine trial was performed over a period of 9 weeks with a vaccination at week 0 and a booster

vaccination at week 6. Blood samples from every ostrich were collected in 5 ml Serum sep clot activator tubes (Vacuette) using 18G x 1" needles (Vacuette) every 3 weeks. Two saliva samples were collected every 3 weeks from each ostrich by swabbing the trachea with sterile swabs (plain, Rayon tipped, sterile swabs with a plastic applicator, Copan). The ostriches were also weighed every 3 weeks during the trial.

It should be mentioned that further bleeds were planned at week 9 and possibly at week 12, but shortly after the week 6 bleed the flock was diagnosed as being positive for AI and was destroyed by the Western Cape Veterinary Services, bringing the trial to a premature end.

4.2.1.2. Preparation of the VR1020 naked DNA vaccine.

The VR1020 naked DNA vaccine was prepared as described in section 3.2.1.2. The vaccine was diluted in PBS buffer (pH 7.2) to a concentration of 100 µg/ml less than 24 hours before vaccination and stored at 4°C until use.

4.2.1.3. Preparation of the VR1020 mucosal DNA vaccine.

A freezer stock of the VR1020 mucosal DNA vaccine prepared in section 3.2.2.2. was streaked onto Petri dishes containing TSA with 100 µg/ml kanamycin. The TSA plates were incubated overnight at 37°C for 16-18 hours. The diagnostic colony PCR described in section 3.2.2.2. and the primer sets in table 3.2 were used to test 10 colonies on the TSA plates and the PCR products visualized on a 1% agarose gel as prepared in section 3.2.1.1. and then visualized under UV light. Two positive colonies were transferred to a 17 x 100 mm polypropylene round-bottom tube containing 5 ml TSB with 100 µg/ml kanamycin and incubated at 37°C shaking at 200 rpm on an orbital shaker (IKA® K260 basic) for 16-18 hours. One millilitre of the overnight culture was then mixed with 49 ml TSB with 100 µg/ml kanamycin and incubated at 37°C, shaking at 200 rpm on an orbital shaker (IKA® K260 basic) to the exponential growth phase. The cultures were centrifuged at 8000 x *g* for 10 min in an Avanti-J-E centrifuge (Beckman). The pellets were washed with 2 ml PBS buffer each and then centrifuged again at 8000 x *g* for 10 min. The pellets were then resuspended in 1 ml PBS (pH 7.5) and kept at 4°C until use. A serial dilution of the prepared recombinant mucosal vaccine was prepared as shown in figure 4.2 and 100 µl of the last three dilutions plated onto each of three Petri dishes containing TSA with 100 µg/ml kanamycin. The plates were incubated at 37°C for 16-18 hours to determine the colony forming units (c.f.u.). To prepare the vaccine for vaccination the recombinant mucosal vaccine was diluted in PBS (pH 7.5) to a dilution of 10^9 c.f.u./ml in a sterilized vaccine serum bottle.

4.2.1.4. Preparation of the Salmonella enterica *serovar* typhimurium *SL3261 control.*

The freezer stock generated in section 3.2.2.1. was inoculated in a 14 ml Falcon® tube containing 5 ml TSB and incubated at 37°C shaking at 200 rpm on an orbital shaker® for 16-18 hours. One

millilitre of the overnight culture was then mixed with 49 ml TSB and incubated at 37°C, shaking at 200 rpm on an orbital shaker® to the exponential growth phase, an OD_{600} of approximately 0.1000. The cells were harvested by centrifugation at 8,000 x *g* for 20 min in an Avanti-J-E centrifuge (Beckman). The pellets were washed with 2 ml PBS buffer each and centrifuged again at 8,000 x *g* for 10 min. The pellet was then dissolved in 1 ml PBS and stored at 4°C until use. A serial dilution of the cells was prepared, as in figure 4.2 and the last three dilutions plated onto Petri dishes containing TSA. The plates were incubated at 37°C for 24 hours, and the c.f.u. determined. The cells were then diluted to 10^9 c.f.u./ml in a sterilized vaccine serum bottle.

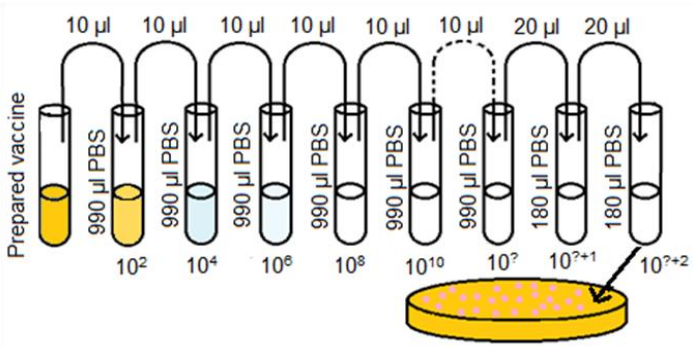

Figure 4.2: Serial dilution of *Salmonella enterica* serovar *typhimurium* SL3261 mucosal vaccines. Mix 10 µl of the 1 ml prepared vaccines with 990 µl PBS to prepare a 10^2 dilution. Transfer 10 µl of the 10^2 dilution to a clean tube containing 990 µl and mix. Repeat this to a desired dilution. Prepare the last dilutions by mixing 20 µl of the $10^?$ dilution with 180 µl for a 10 dilution. Plate 100 µl of the last three dilutions on each of three TSA plates containing the appropriate antibiotic

4.2.2. Analysis of samples collected during the preliminary vaccine trial on a commercial ostrich farm in Oudtshoorn, Western Cape.

4.2.2.1. Processing of blood and saliva samples collected.

The blood samples collected were incubated at 37°C for an hour, and then stored overnight at 4°C. The samples were centrifuged at 140 x *g* for 20 min and the serum transferred into an Eppendorf tube and stored at -20°C until use. One of the swab samples from each ostrich was stored at 4°C until used for analysis of the ostrich immune response using an ELISA. The other swab sample of each ostrich was immersed in 200 µl PBS (pH 7.2) and briefly vortexed before the swab was removed. The samples were then stored at 4°C until analysed for ostrich *Mycoplasma* and *Salmonella* infection after which it was stored at -20°C.

4.2.2.2. Analysis of saliva samples for Mycoplasma *infection.*

To test for *Mycoplasma* infection three PCR reactions were run on each sample testing for each of the three ostrich specific mycoplasmas; *Mycoplasma struthionis*, *Ms02*, and *Mycoplasma nasistruthionis*. The PCR reaction mixture contained 2 µl of a 10x reaction buffer, 1.6 µl of a 25 mM

$MgCl_2$, 0.4 µl of each primer (20 pmol/µl), 0.8 µl dNTP mix (containing 5 mM of each dATP, dCTP, dGTP and dTTP) (Bioline), 0.025 U/µl Taq DNA polymerase (Supertherm polymerase, JMR Holdings), 12.7 µl autoclaved Milli-Q water and 1 µl of the swab sample prepared in section 4.2.2.1. The primers used to test for each of the ostrich mycoplasmas are listed in Table 4.1 (originally developed by Botes, 2005b). The PCR was performed as follows: 30 cycles each consisting of a 30 sec denaturation step at 94°C, a 15 sec annealing step at 57°C and a 1 min extension step at 72°C; followed by a final extension cycle at 72°C for 6 min. The PCR product was then electrophoresed as described in section 3.2.1.1 and visualized under UV.

Table 4.1: Primer sets for identification of the ostrich-specific *Mycoplasmas*

Mycoplasma species	Primer	Sequence	Amplified product size (bp)
Mycoplasma struthionis	Ms01 Z (F)	5'-AACATTAGTTAATGCCGGATACG-3'	499
	Ms01 D (R)	5'-GCCAGTATCCAAAGCGAGCC-3'	
Ms02	Ms02 H (F)	5'-AATATAAAAGGAGCGTTTGC-3'	287
	Ms02 A (R)	5'-AAGGCAATAGCATTTCCTCTACT-3'	
Mycoplasma nasistruthionis	Ms03 A (F)	5'-AGTGCTAATGCCGGATACTTATA-3'	521
	Ms03 C (R)	5'-CGTTAACCTCTATACAATTCTAGCG-3'	

4.2.2.3. Analysis of saliva samples for Salmonella *infection.*

The multiplex PCR published by Lim *et al.* (2003) was used to test for various *Salmonella spp*. infection. The PCR reaction mixture contained 2.5 µl of a 10x reaction buffer, 2.5 µl of a 25 mM $MgCl_2$, 1.25 µl of each of the 6 primers listed in Table 4.2 (20 pmol/µl), 1 µl dNTP mix (containing 5 mM of each dATP, dCTP, dGTP and dTTP) (Bioline), 5 µl Taq DNA polymerase (Supertherm polymerase, JMR Holdings), and 2.5 µl autoclaved Milli-Q water. One microlitre of the bacterial DNA was amplified with the first denaturation cycle at 95°C for 3 min; followed by 30 cycles consisting of a 1 min denaturation step at 95°C, a 30 sec annealing step at 65°C and a 30 sec extension step at 72°C; then followed by a final 1 min extension cycle at 72°C. The PCR product was analysed on a 2% agarose gel prepared as in section 3.2.2.2. and visualized under UV light.

Table 4.2: *Salmonella enterica* serovar *typhimurium* multiplex primer set

Primer	Sequence	Amplified product size (bp)
Rfbj-s	5'-CCAGCACCAGTTCCAACTTGATAC-3'	663
Rfbj-as	5'-GGCTTCCGGCTTTATTGGTAAGCA-3'	
Fljb-s	5'-ATAGCCATCTTTACCAGTTCCCCC-3'	183
Fljb-as	5'-GCTGCAACTGTTACAGGATATGCC-3'	
Flic-s	5'-ACGAATGGTACGGCTTCTGTAACC-3'	526
Flic-as	5'-TACCGTCGATAGTAACGACTTCGG-3'	

4.2.3. Evaluation of immune responses elicited in preliminary vaccine trial

4.2.3.1. Isolation of OppA protein for ELISA plate coating

The OppA protein of *M. struthionis* was used to coat microtitre plates for ELISA analysis of immune responses produced by each of the vaccines. To obtain the OppA protein it first had to be expressed. The O*ppA* gene fused to GST was previously cloned into the pGEX expression vector (Brandt 2012). Brandt (2012) showed that OppA could be expressed as a Glutathione S-transferase (GST)-tagged fusion protein. The pGEX expression vector plasmid containing the OppA gene was transformed into BL21(DE3)pLysS cells and freezer stocks stored at -80°C. As controls BL21(DE3)pLysS cells that contain only the pGEX plasmid that had been stored as freezer stocks were used. Both of these freezer stocks were streaked separately onto Petri dishes containing LB agar and ampicillin (100 µg/ml, Sigma-Aldrich) and incubated at 37°C for 16-18 hours. A diagnostic colony PCR was run on selected colonies as described by section 3.2.1.1. using the primers listed in Table 4.3 to confirm the presence of the *oppA* gene. A 14 ml Falcon® tube containing 5 ml TB medium (12 g/L Bacto-tryptone (Biolab, Merck), 24 g/L Bacto-yeast extract (Biolab, Merck), 4 ml/L of 2 M Glycerol and 100ml/L of 1 M Phosphate buffer (pH 7.8), glucose (20% m/v), chloramphenicol (34 µg/ml, Sigma-Aldrich) and ampicillin (100 µg/ml, Sigma-Aldrich) was inoculated with two of the colonies that tested positive for pGEX + o*ppA*. Another 14 ml Falcon® tube containing 5 ml TB medium, glucose (20% m/v), chloroamphenicol (34 µg/ml, Sigma-Aldrich) and ampicillin (100 µg/ml, Sigma-Aldrich) was inoculated with two pGEX only colonies. The 14 ml Falcon® tube were shaken at 200 rpm on an orbital shaker ® at 37°C for 16-18 hours. For protein expression, 2 ml overnight culture was inoculated with 100 ml TB medium containing, glucose (20% m/v), chloroamphenicol (34 µg/ml, Sigma-Aldrich) and ampicillin (100 µg/ml, Sigma-Aldrich). The cultures were incubated at 37°C to an OD_{600} of 0.6, and then 0.1 M (IPTG) (400 µl/100 ml) was added for recombinant gene expression. The cells were harvested after 6-8 hours by centrifugation at 10 000 x *g* for 15 min. The pellet was resuspended in TEN 50 buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 50 mM NaCl; 0.1% Triton X-100; 0.2 M DTT and 10% (v/v) glycerol) and stored at -80°C until use.

Table 4.3: Primer sets used to test pGEX containing *OppA* and the pGEX control and the expected PCR product sizes after PCR.

BL21 (DE3)pLys5 cells	Primer	Sequence	Amplified product size (bp)
pGEX + *oppA*	Pgex_F	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	2979
	Pgex_R	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	
pGEX only	Pgex_F	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	150
	Pgex_R	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	

The recombinant protein was isolated from the cells suspended in TEN50 by gravity flow through a glutathione agarose gel (Sigma-Aldrich). The glutathione agarose lyophilized powder was swelled in MilliQ water at 200 ml/g for 30 min at room temperature then at 4°C overnight. The final bed

volume [$\pi r^2 h = \pi \times (0.18)^2 \times 10$] is 10 ml. The gel was then washed with 10 volumes of water before it was de-aerated and the slurry poured into the column. The resin was then equilibrated with 10 column volumes of equilibration buffer (PBS buffer (pH 7.4)). The harvested cells were prepared for loading onto the column by freeze thawing as described and then the cell suspension was sonicated for 2 sec (repeated 5 times) and then incubated on ice for 20 sec. The cell suspension was then centrifuged at 4°C, 10 000 x *g* for 10 min. The clarified supernatant was subsequently loaded onto the column and passed through the resin 5 times, before it was incubated on the column for 1 hour to allow the GST-tagged OppA protein to bind to the glutathione on the resin. The resin was then washed with 4 column volumes PBS-T (PBS (pH 7.2) and 1% Triton X-100) and followed by 4 column volumes of PBS buffer (pH 7.2). The GST-tagged OppA protein was finally eluted with 3 column volumes elution buffer (10 mM reduced glutathione and 50 mM Tris-HCl, pH 9.5), by collecting 1 ml fractions in Eppendorf tubes. The resin was washed with 5 column volumes Cleansing buffer 1 (100 mM Borate buffer (pH 8.5) and 500 mM NaCl) followed by 5 column volumes MilliQ water. Five column volumes of cleansing buffer 2 (100 mM acetate buffer (pH 4.5) and 500 mM NaCl) were then used followed by 5 column volumes of MilliQ water, before the resin was stored in storage buffer (2 M NaCl, 0.2% thiomersal).

The protein concentration in each fraction was determined using the Micro Bradford method as follows. The Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) Ethanol, 8.5% (v/v) Phosphoric acid) was prepared as described by (Bradford 1976). A protein standard series was prepared using BSA and the elution buffer to final concentrations 0, 0.25, 0.50, 0.75, 1, 1.25 and 1.5 mg/ml. Five microlitre of the BSA standards and fractions were pipetted into a 96-well microtitre plate (Microlon®, Greiner bio-one) in duplicate and 250 µl of Bradford reagent was added. After the plate was incubated at room temperature for 2 min the absorbance was read at 620 nm on a Labysystems Multiscan MS microtiter plate reader.

To confirm that the GST-tagged OppA protein was isolated, the final protein fraction was electrophoresed on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. A 10% resolving gel was prepared by mixing 8 ml resolving buffer (375 mM Tris-HCl (pH 8.8) containing 0.1% (w/v) SDS), 4 ml resolving monomer (30%T, 2.7% C), 30 µL TEMED (N,N,N'N'-tetramethylene diamine) and 36 µL of 20% (w/v) ammonium persulfate and allowed to polymerase for 20 min between two glass plates separated by 1.5 mm spacers. A 4.5% stacking gel was then prepared by mixing 4.25 ml stacking buffer (125 mM Tris-HCl (pH 6.8) containing 0.1% (w/v) SDS), 0.75 ml stacking monomer (30%T, 2.7% C), 15 µL TEMED (N,N,N'N'-tetramethylene diamine) and 30 µL 20% (w/v) ammonium persulfate. The gel was allowed to polymerize overnight at 4°C. The gel was then setup in a gel apparatus with electrode buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS, pH 8.3). Each of the samples was mixed with an equal volume of reducing treatment buffer (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, pH 6.8) and 0.1 volumes bromophenol blue (0.1% (w/v) bromophenol blue in 100 mM NaOH). After

the mixture was incubated in a boiling water bath for 2 min it was kept on ice until use. Twenty microlitres of each sample and 5 µl Pageruler™ Unstained Protein Ladder (Fermentas) were loaded on the SDS-PAGE gel and electrophoresed at a constant current of 20 mA. The gel was stained with staining solution (0.125% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid) at 37°C for 1 hour. The gel was then destained in destain solution 1 (50% (v/v) methanol and 10% (v/v) glacial acetic acid) for 1 hour at 37°C before destaining the gel overnight in destain solution 2 (5% (v/v) methanol and 7% (v/v) glacial acetic acid) at room temperature.

The GST can be cleaved from the protein using site-specific proteases, but in a study performed in this laboratory it was shown in a western blot that the antibodies raised against OppA in a rabbit did recognize the fusion protein and only recognised OppA and not GST (results not shown). The GST was therefore not cleaved from the OppA protein for the ELISAs used to test the immune response of the ostriches against the recombinant vaccine. The GST-tagged OppA fusion protein will be referred to as OppA when referring to coating of ELISA plates hereafter in this chapter.

4.2.3.2. Isolation of Salmonella enterica *serovar* typhimurium *SL3261 lipopolysaccharide (LPS) for ELISA plate coating*

The hot phenol/water extraction method was used to extract LPS from *Salmonella enterica* serovar *typhimurium SL3261* (Westphal 1965, De Castro *et al.* 2010). A loop was used to transfer cells from the freezer stock of the *Salmonella enterica* serovar *typhimurium SL3261*, prepared in section 3.2.2.2., to 4 round-bottom polypropylene tubes each containing 5 ml TSB and incubated at 37°C for 16-18 hours, shaking at 200 rpm. Two millilitres of the overnight solutions were used to inoculate each of 5 Erlenmeyer flasks containing 100 ml TSB. The flasks were incubated at 37°C for 16-18 hours, shaking at 200 rpm. The OD_{600} was measured and the cells harvested by centrifugation at 9000 x *g* for 20 min at 4°C. The cells were washed twice with 200 ml PBS (pH 7.2). The cells were then washed 4 times with 50 ml -20°C acetone by adding the acetone and incubated at -20°C for 20 min before decanting and adding the acetone for the next round. The pellet was then air dried in a fume hood. The dried cells were then placed in a 65-70°C and prewarmed 90% phenol was added so that the cells make up 20% of final volume and mixed gently with a glass rod. The mixture was stirred at 65-70°C for 30 min. The cells were then centrifuged at 10 000 x *g* for 30 min for 4°C. The water phase was transferred to a glass vial and the above steps repeated with the remaining solution. The water phases were combined and dialysed against distilled water for 48 hours with 4 water changes and then freeze dried with liquid nitrogen and stored at 4°C.

Confirmation of LPS isolation was performed using silver staining of SDS-PAGE gels (Chevallet, Luche & Rabilloud 2006). An SDS-PAGE gel was prepared as in 4.2.3.1., the isolated LPS and a

Salmonella enterica serovar *typhimurium* LPS (Sigma-Aldrich) control sample was loaded onto the gel. The gel was electrophoresed at 15 mA to run into the stacking gel and at 25 mA once the marker had reached the resolving gel. The remainder of the steps were performed in flat Pyrex dishes on an orbital shaker. Firstly, the gel was fixed three times in a fixation solution (30% ethanol, 10% acetic acid) for 30 min. The gel was washed twice in 20% ethanol for 10 min and then twice in MilliQ water for 10 min. In a new glass bowl, the gel was sensitized in 0.02% (w/v) sodium thiosulfate pentahydrate for 1 min before it was washed twice for 1 min in MilliQ water. The gel was then impregnated in 12 mM (w/v) silver nitrate ($AgNO_3$) for 20 min whereafter it was washed in MilliQ water for 5-10 sec. Using developer solution (3% (w/v) Potassium carbonate, 125 µl 10% Sodium thiosulfate pentahydrate and 250 µl formaldehyde per 1 L solution) the gel was developed for 10 min before it was stopped for 30 min using stopping solution (4% (w/v) Tris and 2% (v/v) acetic acid). The gel was then stored in MilliQ water.

4.2.3.3. Biotinylation of rabbit anti-ostrich antibodies

Three different antibodies were biotinylated and used as secondary antibodies in the ELISAs used to test the immune response elicited in the Oudtshoorn vaccine trial. The first antibody; rabbit anti-ostrich immunoglobulin (Ig); was used to test the immune response in ostrich serum. The antibodies were produced in rabbits immunized with ostrich Ig (Van Tonder, A. & Bellstedt, D.U. 2009). The other two antibodies were rabbit anti-ostrich immunoglobulin A (IgA) and were used to test the mucosal immune response in ostrich saliva. These antibodies were produced in rabbits immunized with ostrich IgA without the secretory component (protein 1) and ostrich IgA heavy chain with its secretory component (protein 2) (Botes 2004). All three antibodies were firstly isolated from rabbit serum by ammonium sulphate precipitation as follows. Two millilitres of rabbit serum were mixed gently by inversion with 4 ml PBS (pH 7.2) and 6 ml saturated ammonium sulphate (pH 7.2). The mixture was incubated at 4°C for 20 min, then centrifuged at 17,640 x *g* for 20 min. The pellet dissolved in 4 ml PBS (pH 7.2) and 4 ml of saturated $(NH_4)_2SO_4$ solution was added and incubated at 4°C for 20 min. The mixture was then again centrifuged at 17,640 x *g* for 20 min and the pellet finally dissolved in 2 ml PBS (pH7.2). The mixture was then dialysed against carbonate buffer (0.1 M $NaHCO_3$, pH 8.3) at 4°C overnight in 10 mm x 6 mm cellulose membrane dialysis tubing (Sigma Aldrich), with a buffer change after 5 hours. After overnight dialysis the volume of the mixture in the dialysis tubing was measured and the concentration measured at 280 nm with a Nanodrop® ND 1000 spectrophotometer.

The isolated immunoglobulin was diluted to a concentration of 5 mg/ml solution in 0.1 M $NaHCO_3$ (pH 8.3) in a pear bottom flask. A volume of Biotin reagent (2 mg Biotinamidocaproate N-hydroxysuccinimide ester /ml N,N-Dimethylformamide (DMF)) which was one quarter of the total volume of the above dialysate, i.e. the isolated immunoglobulin, was added dropwise and gently stirred at room temperature for 2 hours. The mixture was then dialysed against PBS buffer (pH 7.2)

overnight at 4°C in 25 mm x 16 mm cellulose membrane dialysis tubing (Sigma Aldrich), with a buffer change after 5 hours. The biotinylated antibodies were then mixed with an equal volume of glycerol and stored at -20°C until use.

4.2.3.4. Ostrich immune response measurement against OppA

ELISA optimization:

The immune response elicited in the ostriches was assessed by ELISA. Before this could be done, however, an optimization ELISA was performed using one immunized ostrich as follows. The wells of a 96-well ELISA plate (Maxisorp, Nunc) were prepared by coating columns 1 to 11 with OppA protein diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6). Rows A and B were coated with 1 µg/ml OppA, rows C and D with 2 µg/ml OppA, rows E and F with 5 µg/ml OppA, rows G and H with 10 µg/ml OppA. Column 12 was left uncoated to test the specificity of the assay. The plate was incubated overnight at 4°C. Non-specific binding to the plate was then blocked with 200 µl caseïen buffer (154 mM NaCl, 10 mM Tris-HCl, 0.02% thiomersal and 0.5 % caseïen, pH 7.6) per well for 1-2 hours at 37°C. The wells were subsequently washed 6 times with PBS-tween 20 (PBS buffer (pH 7.2) containing 0.1% (v/v) tween 20). A 1:20 dilution of the ostrich serum was prepared in caseïen-tween 20 (caseïen buffer (pH 7.6) containing 0.1% (v/v) tween 20) and 200 µl pipetted into column 2 and 100 µl into column 12. To columns 1 and 3-11, 100 µl of caseïen-tween 20 (pH 7.6) was added. To make a serial dilution from column 2 to 11, 100 µl of column 2 was mixed with column 3, then 100 µl of column 3 was mixed with column 4, this was done until column 11 was reached, after which 100 µl was discarded from each well in column 11 to leave only 100 µl in each well. The final volume in each well was therefore 100 µl. The plate was then incubated at 37°C for 1 hour. After the plate was again washed 6 times with PBS-tween 20 (pH7.2), 100 µl of the biotinylated rabbit anti-ostrich antibodies prepared in section 4.2.3.2., that was diluted 1:100 in caseïen-tween 20, was added to each well and the plate incubated at 37°C for 1 hour. The plate was washed 6 times with PBS-tween 20 then incubated at 37°C for 1 hour with 100 µl Streptavidin Horseradish Peroxidase (HRP) Conjugate (Invitrogen) diluted 1:100 in caseïen-tween 20. A 1:40 dilution of the Streptavidin-HRP was first prepared by mixing 2 ml of the Streptavidin-HRP ready made sample with 38 ml caseïen buffer (pH 7.6) and 40 ml glycerol, the mixture was stored at -20°C until use. The plate was then washed again with PBS-tween 20 6 times before 100 µl of substrate (0.006 g ABTS, 12 ml citrate buffer (100 mM citric acid monohydrate and 100 mM tri-sodium citrate dehydrate, pH 5) and 6 µl H_2O_2) was added and the plate incubated at 37°C. The absorbance was measured at 405 nm every 5 min for 30 min on a Labysystems Multiscan MS microtiter plate reader. The optimization ELISA was also done with coating concentrations of 0.1, 0.2, 0.5 and 1 µg/ml OppA.

ELISA validation:

The serum of a rabbit immunized with the OppA protein was used to test if the OppA used for coating can be recognised in the ELISA. The wells of a 96-well ELISA plate (Maxisorp, Nunc) were prepared by coating column 1 to 11 with 1 µg/ml of OppA protein diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6). Column 12 was left uncoated to test the specificity of the assay. The plate was incubated overnight at 4°C. The plate was then blocked with 200 µl caseïen buffer (154 mM NaCl, 10 mM Tris-HCl, 0.02% thiomersal and 0.5% caseïen, pH 7.6) per well for 1-2 hours at 37°C. The wells were washed 3 times with PBS-tween 20 (PBS (pH 7.2) containing 0.1% (v/v) Tween 20). A 1:20 dilution of the serum was prepared in caseïen-tween 20 (caseïen buffer (pH 7.6) containing 0.1% (v/v) Tween 20) and 200 µl pipetted into column 2; rows B, D and F; and 100 µl into column 12. To columns 1 and 3-11, 100 µl of caseïen-tween 20 was added. To make a serial dilution across columns 2 to 11, 100 µl of column 2; rows B, D and F; was mixed with column 3; rows B, D and F; then 100 µl of column 3; rows B, D and F; was mixed with column 4; rows B, D and F; this was done until column 11; rows B, D and F; was reached. The serial dilution was continued in rows C, E and G. The final volume in each well was 100 µl. The plate was then incubated at 37°C for 1 hour. After the plate washed again 3 times with PBS-tween 20 (pH 7.2), 100 µl goat anti-rabbit antibodies were diluted 1:500 in caseïen-tween 20, and was added to each well and the plate incubated at 37°C for 1 hour. The plate was washed again 3 times with PBS-tween 20 (pH 7.2) before 100 µl rabbit peroxydase anti-peroxidase (PAP) antibodies was diluted 1:1000 in caseïen-tween 20, was added to each well and the plate incubated at 37°C for 1 hour. The plate was then washed again with PBS-tween 20 (pH 7.2) 3 times before 100 µl of substrate solution (0.006 g 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 12 ml citrate buffer (100 mM citric acid monohydrate and 100 mM tri-sodium citrate dehydrate, pH 5) and 6 µl H_2O_2) was added and the plate incubated at 37°C. The absorbance was measured at 405 nm every 5 min after addition for 30 min on a Labysystems Multiscan MS microtiter plate reader. The optimization ELISA was also done with coating concentrations of 0.1, 0.2, 0.5 and 1 µg/ml OppA.

Humoral immune response of ostriches to *Mycoplasma struthionis*:

To assess the humoral immune response of the ostriches to the OppA protein elicited by the DNA vaccines the following ELISA was used. The method is described above under “ELISA optimization” with the following modifications. Column 1 and Columns 2-12 rows A, C, E and G were coated with 1 µg/ml OppA and Columns 2-12 rows B, D, F, H were left uncoated. A serial dilution of the serum was not made across the plate, instead the serum collected at week 0, week 3 and week 6 from each ostrich was only diluted 1:100 and 100 µl of this dilution pipetted into a coated wells in duplicate and in uncoated wells.

Mucosal immune response of ostriches to *Mycoplasma struthionis*:

The second swab sample obtained from each ostriches in the vaccination trial was stored as described in section 4.2.1.3. but was then immersed in PBS (pH 7.2) containing 0.1%(w/v) Triton X-100. The immune responses of the ostriches against the OppA protein were assessed using the ELISA protocols described in section 4.2.3.3, with two exceptions. The ostrich swab samples were diluted 1:20 in caseïen-tween 20 for incubation instead of 1:100 used for the ostrich serum. The second change was that instead of biotinylated rabbit anti-ostrich immunoglobulin being used, biotinylated rabbit anti-ostrich IgA protein 1 and biotinylated rabbit anti-ostrich IgA protein 2 were used separately for each sample, respectively.

4.2.3.5. Ostrich immune response measurement against Salmonella enterica *serovar* typhimurium SL3261 *LPS*

ELISA optimization:

The immune responses of the ostriches to the *Salmonella enterica* serovar *typhimurium SL3261* LPS was assessed by means of an ELISA very similar to that for the assessment of the immune response to OppA. The method described in section 4.2.3.3 was used with the following modifications. For Rows A and B, Column 1 to 5 was coated with 0.5 µg/ml LPS dissolved in PBS (pH 7.2) and Column 7 to 11 was coated with 0.5 µg/ml LPS dissolved in carbonate buffer (pH 9.6). For Rows C and D, Column 1 to 5 was coated with 1.0 µg/ml LPS dissolved in PBS (pH 7.2) and Column 7 to 11 was coated with 0.5 µg/ml LPS dissolved in carbonate buffer (pH 9.6). For Rows E and F, Column 1 to 5 was coated with 3 µg/ml LPS dissolved in PBS (pH 7.2) and Column 7 to 11 was coated with 0.5 µg/ml LPS dissolved in carbonate buffer (pH 9.6). For Rows G and H, Column 1 to 5 was coated with 5.0 µg/ml LPS dissolved in PBS (pH 7.2) and Columns 7 to 11 were coated with 0.5 µg/ml LPS dissolved in carbonate buffer (pH 9.6). Columns 6 and 12 were left uncoated. A serial dilution of the serum was made from Column 2 to 5 and again in Columns 8 to 11 starting with a dilution of 1/40. Rows A, C, E and G received sera from week 0 and Rows B, D, F and H received sera from week 3. The rest of the ELISA procedure was the same as described in section 4.2.3.3.

Humoral immune response of the ostriches to *Salmonella enterica* serovar *typhimurium* SL3261:

To test the immune response of the ostriches to *Salmonella enterica* serovar *typhimurium SL3261* an ELISA was used. The method in section 4.2.3.3 was used with the following modifications. Column 1 and Column 2-12 rows A, C, E and G was coated with 5 µg/ml *Salmonella* LPS and Columns 2-12 rows B, D, F, H was left uncoated. A serial dilution of the serum was not made across the plate, instead the serum of week 0, week 3 and week 6 for each ostrich was a diluted

1:100 and 100 µl pipetted into coated wells in duplicate and in uncoated wells. Biotinylated rabbit anti-ostrich immunoglobulin antibody was used as secondary antibody.

Mucosal immune response of the ostriches to *Salmonella enterica* serovar *typhimurium* SL3261:

The swab samples obtained from the ostriches in the vaccination trial were prepared as described in section 4.2.1.3., but the samples were immersed in PBS (pH 7.2) containing 0.1%(w/v) Triton X-100. The immune responses of the ostriches against *Salmonella enterica* serovar *typhimurium* SL3261 were assessed using the ELISA protocols described in section 4.2.3.3, with two exceptions. The ostrich swab samples were diluted 1:20 in caseïen-tween 20 for incubation instead of the 1:100 used for the ostrich serum. The second change was that instead of biotinylated rabbit anti-ostrich immunoglobulins, biotinylated rabbit anti-ostrich IgA protein 1 and biotinylated rabbit anti-ostrich IgA protein 2 were used separately for each sample.

4.2.3.6. Statistical analysis of ELISA data

The immune responses as measured by ELISA after vaccination with the VR1020 naked DNA vaccine and the VR1020 mucosal DNA vaccine were analysed using ANOVA in the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

4.3. Results

4.3.1. Analysis of samples collected during preliminary vaccine trial on a commercial ostrich farm in Oudtshoorn, Western Cape.

The results of the tests for the detection of ostrich *Mycoplasma* and *Salmonella* infection in the ostriches prior to and during the vaccination trial are shown in table 4.4. All the ostriches showed the presence of Salmonella infections at week 0, which were cleared by week 6 after treatment with Tenaline® LA. Only a few *Ms02* and *Mycoplasma nasistruthionis* infections were found.

4.3.2. Evaluation of immune response elicited in preliminary vaccine trial

4.3.2.1. Isolation of OppA protein for ELISA plate coating

The agarose gel in figure 4.3 shows a band at approximately 3000 bp which corresponds with the expected size of the BL21(DE3)pLysS cells successfully transformed with pGEX containing the *oppA* gene and a band at appoximately 150 bp corresponding to the expected size of BL21(DE3)pLysS cells successfully transformed with the pGEX vector only. The concentrations of the fractions containing the isolated GST OppA fusion protein obtained from the glutathione agarose gel are shown in figure 4.4. The fraction with the highest protein concentration was fraction 6, with a concentration of 0.485 mg/ml. The results of of the SDS-PAGE separation of the fractions that contained the highest protein concentration are shown in figure 4.5. The SDS-PAGE

gel shows bands at approximately 150 kDa and 30 kDa which correspond to the expected size of the GST OppA fusion protein (about 130 kDa) and the GST protein only (26 kDa), respectively (Brandt 2012).

4.3.2.2. Isolation of Salmonella enterica *serovar* typhimurium *SL3261 lipopolysaccharide (LPS) for ELISA plate coating*

The isolated *Salmonella enterica* serovar *typhimurium* SL3261 LPS was separated on an SDS-PAGE gel and visualized with silver staining shown in figure 4.6A. In general, the staining of the bands was not very strong as a result of which the bands on the photograph are not all clearly visible. However, although the bands obtained in the lanes in which the isolated LPS samples were loaded was weaker, they were similar to those of the commercial *Salmonella enterica* serovar *typhimurium* LPS. The isolated LPS showed a ladder-like pattern in the low molecular weight range which is characteristic of LPS molecules, showing different amounts of repetitive O-antigen groups. Althought the commercial *Salmonella enterica* serovar *typhimurium* LPS shows different bands in the higher molecular weight range, it also shows the characteristic ladder-like banding in the low molecular weight range.

4.3.2.3. Ostrich immune responses measured against OppA

ELISA optimization:

The optimal OppA protein coating concentration for coating was found to be 1 µg/ml. When this concentration of OppA was used for coating the highest absorbance value in the ELISA of the serum sample from week 3 (1.658) was obtained whilst the absorbance value in the ELISA obtained with the serum sample from week 0 (0.479) remained relatively low. At a lower coating concentration, the week 3 values were too low whilst at a higher coating concentration the week 0 absorbance values in the ELISA were too high. Results not shown.

ELISA validation:
The results of the ELISA validation showed that the ELISA can be used to measure an immune response against the OppA protein. Sera of a rabbit immunized with purified OppA protein were tested with the ELISA and the results obtained are shown in figure 4.7 showing an increase in titer over the period of 45 days.

Table 4.4: Results of the tests on saliva samples for Salmonella and mycoplasma infections of the ostriches in the preliminary vaccine trial.

Vaccine	Ostrich number	Weight Week 0 (kg)	Infection	Weight Week 3 (kg)	Infection	Weight Week 6 (kg)	Infection
Bacterial Control (*Salmonella enterica* serovar *typhimurium* SL3261) Week 0: 10^9 c.f.u./ml Week 6: 10^9 c.f.u./ml	2700	46.6	*Salmonella*	50.5		51.5	
	2701	45	*Salmonella*	45.4		49.4	
	2702	43	*Mycoplasma nasistruthionis, Salmonella*	43	*Salmonella*	44	*Mycoplasma nasistruthionis*
	2703	38	*Salmonella*	40.6	*Salmonella*	47.6	
	2704	49	*Salmonella*	55.5	*Ms02, Salmonella*	63	
	2705	50	*Salmonella*	46		49	
	2706	39	*Mycoplasma nasistruthionis, Salmonella*	39.2	*Salmonella*	42.4	
	2707	40	*Salmonella*	37.8	*Salmonella*	39.6	
	2708	48	*Mycoplasma nasistruthionis, Salmonella*	42		43.6	
	2709	43	*Salmonella*	44.8	*Ms02, Mycoplasma nasistruthionis*	49.2	*Mycoplasma nasistruthionis*
	2710	38	*Mycoplasma nasistruthionis, Salmonella*	40.2	*Salmonella*	41.8	
	2711	39	*Salmonella*	35.2	*Salmonella*	35.8	
	2712	39	*Salmonella*	37.2	*Ms02, Mycoplasma nasistruthionis, Salmonella*	39.4	
	2713	36	*Salmonella*	33.6	*Ms02, Salmonella*	32.8	
	2714	34	*Salmonella*	34.4	*Ms02, Mycoplasma nasistruthionis, Salmonella*	40.4	
	2715	39	*Salmonella*	35	*Ms02*	40.8	
	2716	42	*Salmonella*	35.6	*Salmonella*	37.8	
	2717	46	*Salmonella*	39.8	*Ms02, Mycoplasma nasistruthionis,*	44.8	

					Salmonella		
	2718	39	*Salmonella*	43.8	*Mycoplasma nasistruthionis, Salmonella*	48.2	
	2719	45	*Salmonella*	44.6		50	
	2720	51	*Salmonella*	56.5	*Salmonella*	64	
	2721	40	*Salmonella*	39.2	*Salmonella*	41.2	
	2722	39	*Salmonella*	43.6	*Salmonella*	45.8	
	2723	42	*Salmonella*	40.2	*Salmonella*	42	
	2724	43	*Salmonella*	42.8	*Salmonella*	48	
	2725	43	*Salmonella*	45.4	*Mycoplasma nasistruthionis, Salmonella*	51.5	
	2726	39	*Salmonella*	40.8	*Salmonella*	47	
	2727	49	*Salmonella*	49.6	*Salmonella*	59	
	2728	47	*Salmonella*	44.4	*Salmonella*	46.6	
	2729	39.8	*Salmonella*	41.6	*Salmonella*	43.2	
Mucosal vaccine (*Salmonella enterica* serovar *typhimurium* SL3261 with VR1020 plasmid Week 0: 10^9 c.f.u./ml Week 6: 10^9 c.f.u./ml	2730	44.1	*Salmonella*	43.2	*Ms02, Mycoplasma nasistruthionis, Salmonella*	41.8	*Mycoplasma nasistruthionis*
	2731	46	*Salmonella*	47.4	*Salmonella*	58	
	2732	41	*Salmonella*	42.8	*Salmonella*	-	
	2733	45	*Salmonella*	46.8	*Salmonella*	51.5	*Mycoplasma nasistruthionis*
	2734	47	*Salmonella*	52	*Salmonella*	62.5	
	2735	35	*Salmonella*	30.4	*Salmonella*	35.4	
	2736	40	*Salmonella*	38.8	*Salmonella*	45	
	2737	41	*Salmonella*	46.4	*Ms02, Mycoplasma nasistruthionis, Salmonella*	50	
	2738	34	*Salmonella*	36.4	*Salmonella*	35	
	2739	38	*Salmonella*	38.2	*Mycoplasma nasistruthionis, Salmonella*	46.6	
	2740	48	*Salmonella*	49.2	*Salmonella*	61	

	2741	40	*Salmonella*	43.8	*Salmonella*	50.5	
	2742	42	*Salmonella*	42.4	*Salmonella*	49.8	
	2743	46	*Salmonella*	44.8	*Salmonella*	48.6	
	2744	36	*Salmonella*	34.8	*Salmonella*	32	*Mycoplasma nasistruthionis*
	2745	49	*Salmonella*	50.5	*Salmonella*	52	
	2746	41	*Salmonella*	41.2	*Salmonella*	53.5	*Mycoplasma nasistruthionis*
	2747	43		44.4		53.5	
	2748	41	*Salmonella*	36.8		-	
	2749	36	*Salmonella*	39.4	*Salmonella*	46.6	*Ms02*
	2750	38		40.2	*Salmonella*	46	
	2751	39	*Salmonella*	34.8		45	
	2752	40	*Salmonella*	39	*Ms02, Salmonella*	47.6	
	2753	42	*Salmonella*	41.6		52.5	
	2754	44	*Salmonella*	40.6	*Salmonella*	47.8	
	2755	44	*Salmonella*	44.4	*Salmonella*	46	*Ms02*
	2756	43	*Salmonella*	45.2	*Ms02, Salmonella*	47.4	
	2757	38		40.6	*Salmonella*	45	
	2758	36	*Salmonella*	40.2	*Salmonella*	47.4	
	2759	43	*Salmonella*	41	*Salmonella*	40.8	
Intramuscular vaccine (VR1020 naked DNA vaccine) Week 0: 100 µg/ml Week 6: 100 µg/ml	2760	40	*Salmonella*	39.4	*Salmonella*	42	
	2761	40	*Salmonella*	42.6	*Salmonella*	44.8	*Ms02*
	2762	38	*Salmonella*	37	*Salmonella*	39.8	
	2763	43	*Salmonella*	39.8	*Ms02, Salmonella*	39.2	
	2764	41		41.2	*Salmonella*	49	
	2765	37	*Salmonella*	37.8	*Ms02, Salmonella*	38.2	
	2766	44	*Salmonella*	40.6	*Salmonella*	45.4	*Ms02*
	2767	40	*Salmonella*	43.2	*Ms02, Salmonella*	47.8	
	2768	39	*Salmonella*	43.2	*Ms02, Salmonella*	46.8	
	2769	39	*Salmonella*	38.8	*Salmonella*	39.2	
	2770	37	*Salmonella*	43.8	*Salmonella*	42	
	2771	48	*Salmonella*	41.4	*Salmonella*	42.8	
	2772	37	*Salmonella*	40.8	*Salmonella*	40.4	*Mycoplasma*

							nasistruthionis
	2773	37	*Salmonella*	37.6	*Salmonella*	33.4	
	2774	43	*Salmonella*	46.6	*Ms02, Salmonella*	45.2	
	2775	39	*Salmonella*	32	*Salmonella*	37.8	*Ms02*
	2776	37	*Salmonella*	37.8	*Salmonella*	40.3	*Ms02*
	2777	39	*Salmonella*	41.4	*Salmonella*	44.4	
	2778	41	*Salmonella*	44.6	*Salmonella*	46	*Ms02*
	2779	37	*Salmonella*	38	*Salmonella*	37	
	2780	40	*Salmonella*	41.8	*Salmonella*	44.2	*Ms02*
	2781	39	*Salmonella*	39.2	*Salmonella*	45.8	
	2782	39	*Salmonella*	37.6	*Salmonella*	-	
	2783	40	*Salmonella*	35	*Salmonella*	38	
	2784	41	*Salmonella*	40.8	*Salmonella*	43.4	
	2785	41	*Salmonella*	42.4	*Salmonella*	48.4	*Mycoplasma nasistruthionis*
	2786	41	*Salmonella*	41	*Salmonella*	42.6	
	2787	39	*Salmonella*	36.2	*Salmonella*	36.6	
	2788	39	*Salmonella*	44.4	*Salmonella*	46.6	
	2789	39	*Salmonella*	40.2	*Salmonella*	47.8	*Ms02*
Control (Nothing injected or administered orally)	2790	38	*Salmonella*	37.8	*Salmonella*	42.4	
	2791	40	*Salmonella*	36.8	*Salmonella*	44.4	*Mycoplasma nasistruthionis*
	2792	36	*Salmonella*	32.4	*Salmonella*	35	
	2793	38	*Salmonella*	34.2	*Ms02, Salmonella*	33.2	
	2794	39	*Salmonella*	39	*Ms02, Mycoplasma nasistruthionis, Salmonella*	-	
	2795	37	*Salmonella*	40.2	*Ms02, Mycoplasma nasistruthionis, Salmonella*	46.6	
	2796	40	*Salmonella*	36.2	*Ms02, Mycoplasma nasistruthionis, Salmonella*	36	
	2797	37		34.4		35.8	
	2798	36	*Salmonella*	41.4	*Salmonella*	48.6	*Ms02*

	2799	36	*Salmonella*	36.4	*Ms02, Salmonella*	38.6	
	2800	37		37	*Ms02, Mycoplasma nasistruthionis*	46.2	*Ms02*
	2801	37	*Salmonella*	33.2	*Salmonella*	38.4	*Ms02*
	2802	36	*Salmonella*	36.4	*Ms02, Mycoplasma nasistruthionis, Salmonella*	35.6	*Mycoplasma nasistruthionis*
	2803	37		40.6	*Ms02, Mycoplasma nasistruthionis, Salmonella*	39.4	*Ms02*
	2804	35		36.6	*Salmonella*	37.2	
	2805	43	*Salmonella*	41	*Salmonella*	48.4	
	2806	38	*Salmonella*	35.4	*Salmonella*	40.2	*Ms02*
	2807	38	*Salmonella*	38.2	*Ms02, Salmonella*	38.4	
	2808	45	*Salmonella*	44.4	*Salmonella*	48	
	2809	37	*Salmonella*	35.4	*Ms02, Mycoplasma nasistruthionis, Salmonella*	38.8	
	2810	38	*Salmonella*	38.6	*Salmonella*	43.8	*Ms02*
	2811	38	*Salmonella*	40.2	*Salmonella*	40.2	
	2812	40	*Salmonella*	43.4	*Salmonella*	43	*Mycoplasma nasistruthionis*
	2813	38	*Salmonella*	37.4	*Salmonella*	38.8	*Ms02, Mycoplasma nasistruthionis*
	2814	37	*Salmonella*	34	*Salmonella*	43	
	2815	36	*Salmonella*	31	*Salmonella*	28.2	*Mycoplasma nasistruthionis*
	2816	42	*Salmonella*	47	*Salmonella*	47.6	
	2817	37	*Salmonella*	39.2	*Salmonella*	36	*Mycoplasma nasistruthionis*
	2818	36	*Salmonella*	40	*Salmonella*	42.6	
	2819	35	*Salmonella*	33	*Salmonella*	36.4	*Mycoplasma nasistruthionis*

[Blank] No infections detected

- No data available as ostriches died

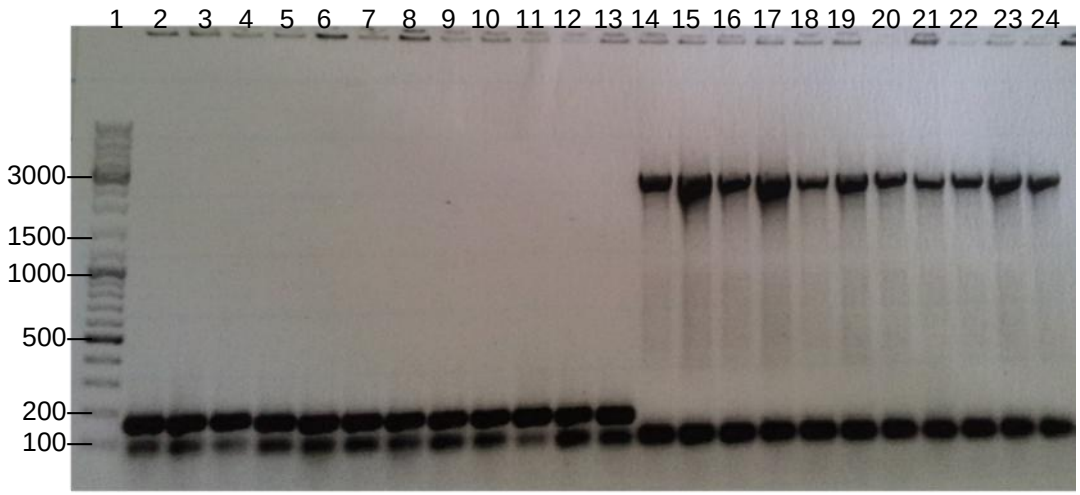

Figure 4.3: Diagnostic colony PCR of transformed cells. i. Lane 1: 5 µl DNA Ladder mix (Generuler™, Fermentas). Lane 2-13: 10 µl PCR product from selected colonies on LB agar plates with BL21(DE3)pLysS transformed with pGEX only cells. Lane 14-24: 10 µl PCR product from selected colonies on LB agar plates with BL21(DE3)pLysS transformed with pGEX containing OppA.

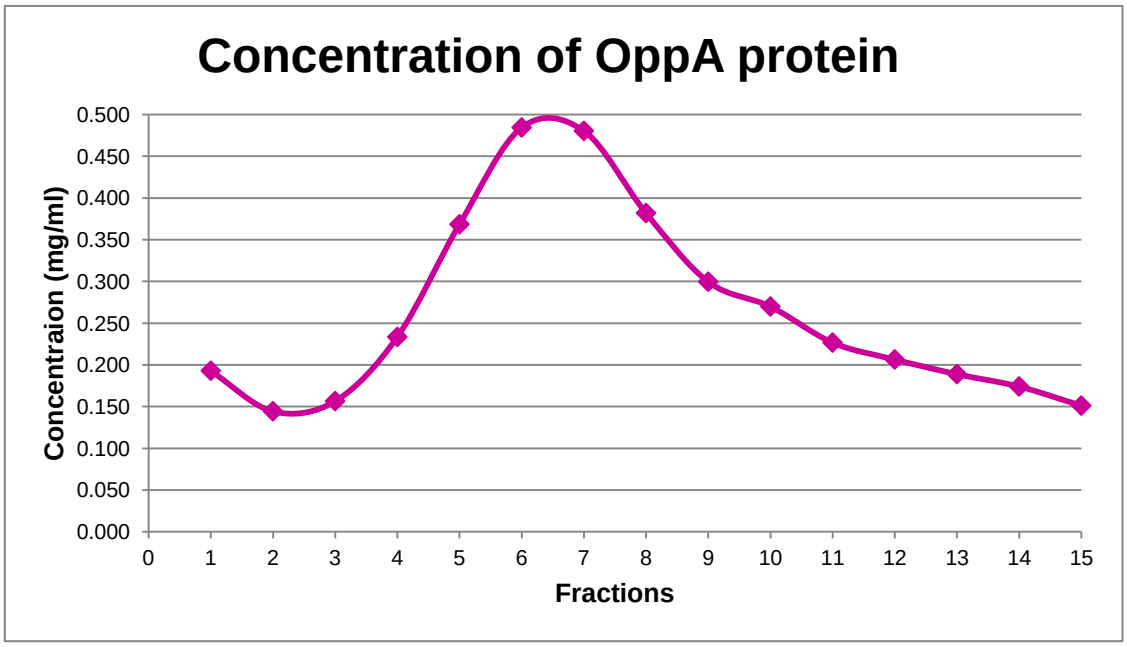

Figure 4.4: Graph of concentration of OppA in the fractions eluted from a glutathione agarose affinity chromatography column.

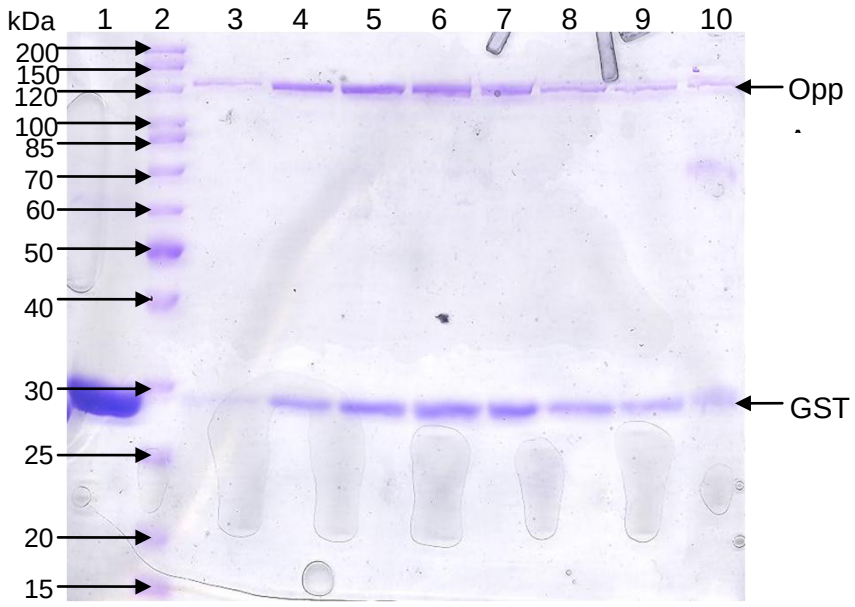

Figure 4.5: SDS PAGE gel of isolated OppA protein. Lane 1: Glutathione S-transferase (GST) Lane 2: 5 µl Protein Ladder (Unstained Pageruler^{TM}) Lane 3 – 10: Fractions 4-11 collected during Glutathione agarose affinity chromatography.

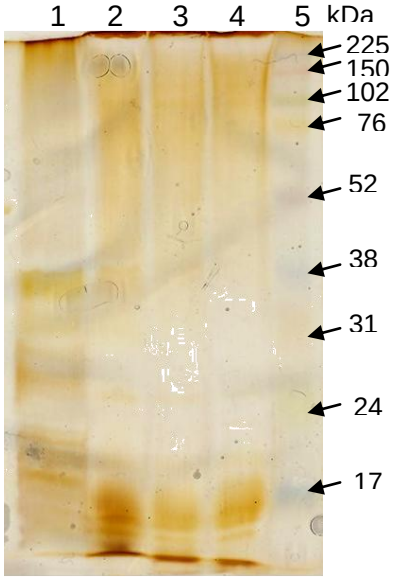

Figure 4.6: Silver stained SDS-PAGE gel to visualize isolated *Salmonella enterica* serovar *typhimurium* SL3261 LPS. Lane 1: 3 µg *Salmonella enterica* serovar *typhimurium* LPS control. Lane 2: 3 µg *Salmonella enterica* serovar *typhimurium* SL3261. Lane 3: 2.5 µg *Salmonella enterica* serovar *typhimurium* SL3261. Lane 4: 2 µg *Salmonella enterica* serovar *typhimurium* SL3261. Lane 5: 5 µl High range rainbow molecular weight marker (GE Healthcare).

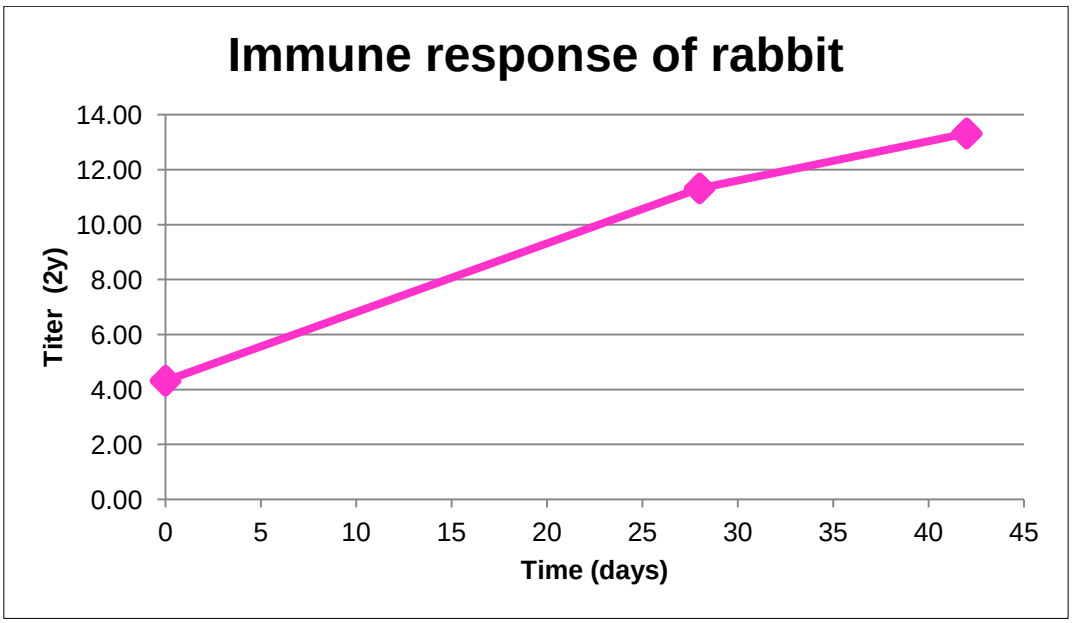

Figure 4.7: Immune response elicited by a rabbit to isolated OppA protein as determined by an ELISA. The rabbit was immunized with purified OppA protein on day 0 and a booster injection was given on day 28. Serum samples collected on day 0, 28 and 42 were tested.

Humoral and mucosal immune responses of ostriches to *Mycoplasma struthionis*

The humoral and mucosal immune responses expressed as absorbance values obtained from the ELISA assessment of the serum and saliva samples were analysed by ANOVA. The results of the analysis are shown in Tables 4.5, 4.6 and 4.7. In view of the large biological variability to be expected from such trials a p-value of 0.05 was accepted as being significant. The ANOVA generates a least significant difference (LSD) value which indicates the smallest difference that is significant between any two points on the graphs. If the effect of a factor i.e., treatment or time was found to be statistically significant, the LSD of that interaction is indicated as a bar on the graphs depicting the averages of the immune response elicited by the ostriches (figures 4.8., 4.9 and 4.10). The same goes for if the interaction between treatment and time was found to be statistically significant. The influence of the vaccine on the weights of the ostriches was also analysed and the results shown in table 4.8 and is shown graphically in figure 4.11. The raw data and a graphical presentation of the immune responses and weights of each bird are shown in Appendix B.

It is a generally accepted rule in ANOVA analysis that if the R squared value is lower than 0.81 and the coefficient of variance (C. V.) is greater than 15%, the variability of the data is too great to make statistically significant deductions (Personal communication with Willem Burger). These values were used as a criterion upon which any deductions were made about the effect of the administration of the vaccines, the *Salmonella* control treatment or the control treatment caused on the observed changes in humoral or mucosal immunity. None of the results obtained from the analysis adhere to these criteria, which means that these results must be treated with caution. It must therefore be concluded that no humoral or mucosal immune response to OppA was induced

as a result of vaccination with the VR1020 mucosal and VR1020 naked DNA vaccines. There are a number of reasons that can explain this and include: that the ostriches were influenced by previous infections with mycoplasmas and *Salmonella*; that the stress of handling has influenced the ability of the ostriches to elicit an immune response; and that the AI may have already compromised the ability of the birds to elicit an immune response. Although the analysis of data of the weights of the ostriches during the trial also has a low R squared value, the C.V. is 16% and this gives an indication that this data can be trusted. The weight increase of these birds was on average lower than what could have been expected for birds of this age so clearly they were influenced by environmental conditions.

Table 4.5: Statistical analysis of humoral immune responses elicited against the different vaccines using anti-ostrich immunoglobulins as detection antibody.

Source	df	SS	MS	Pr>F
Total	359	57.063		
Treatment	3	5.919	1.973	0.0000
Time	2	2.416	1.208	0.0002
Treatment x Time interaction	6	1.361	0.227	0.1284
Residual	348	47.367	0.136	

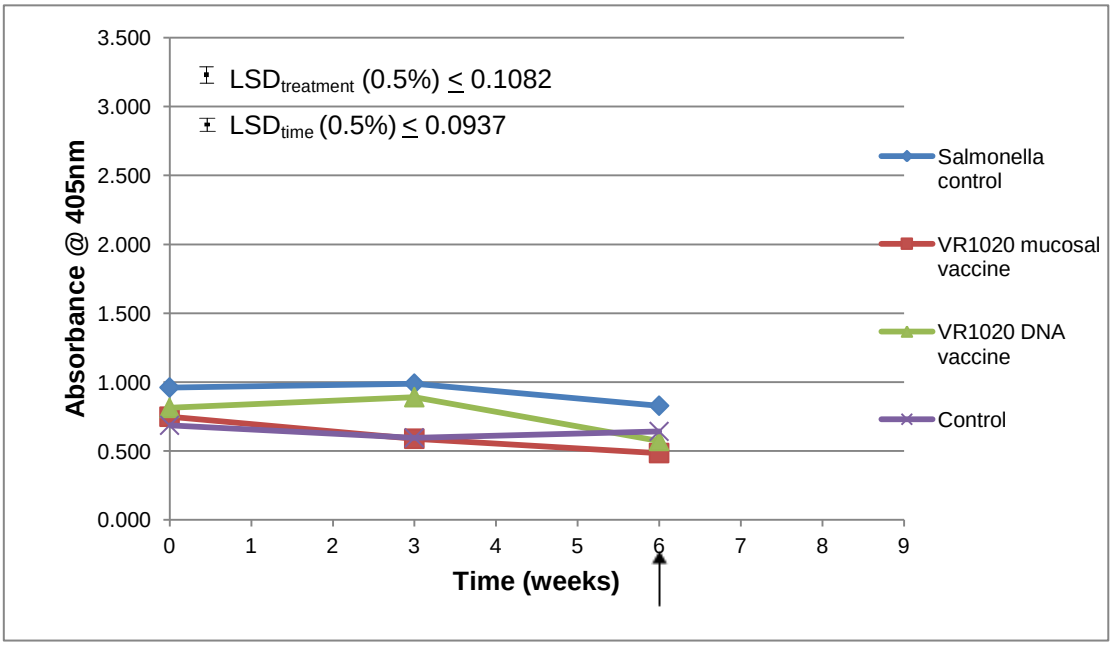

Figure 4.8: Averages of the humoral immune responses elicited in each vaccine group as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich immunoglobulins as detection antibody. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ VR1020 mucosal DNA vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the naked VR1020 recombinant vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

Table 4.6: Statistical analysis of mucosal immune response against the different vaccines using anti-IgA protein 1 as the secondary antibody

Source	df	SS	MS	Pr>F
Total	359	0.050		
Treatment	3	0.003	0.001	0.0000
Time	2	0.000	0.000	0.2474
Treatment x Time interaction	6	0.001	0.000	0.3123
Residual	348	0.046	0.000	

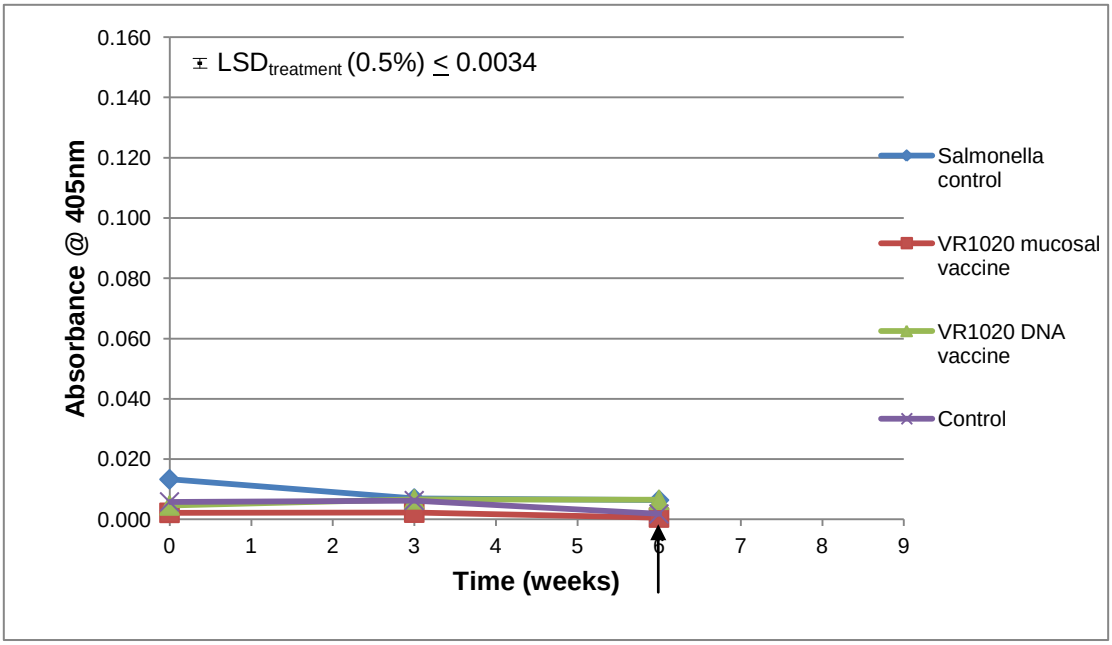

Figure 4.9: Averages of the mucosal immune responses elicited in each vaccine group as determined by an ELISA using 1 µg/ml OppA for coating, saliva collected and rabbit anti-ostrich IgA protein 1 as detection antibody. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ mucosal VR1020 vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the VR1020 naked DNA vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

Table 4.7: Statistical analysis of mucosal immune response measured against the OppA protein using anti-IgA protein 2 as the secondary antibody

Source	df	SS	MS	Pr>F
Total	359	0.063		
Treatment	3	0.001	0.000	0.2527
Time	2	0.000	0.000	0.5329
Treatment x Time interaction	6	0.001	0.000	0.6294
Residual	348	0.061	0.000	

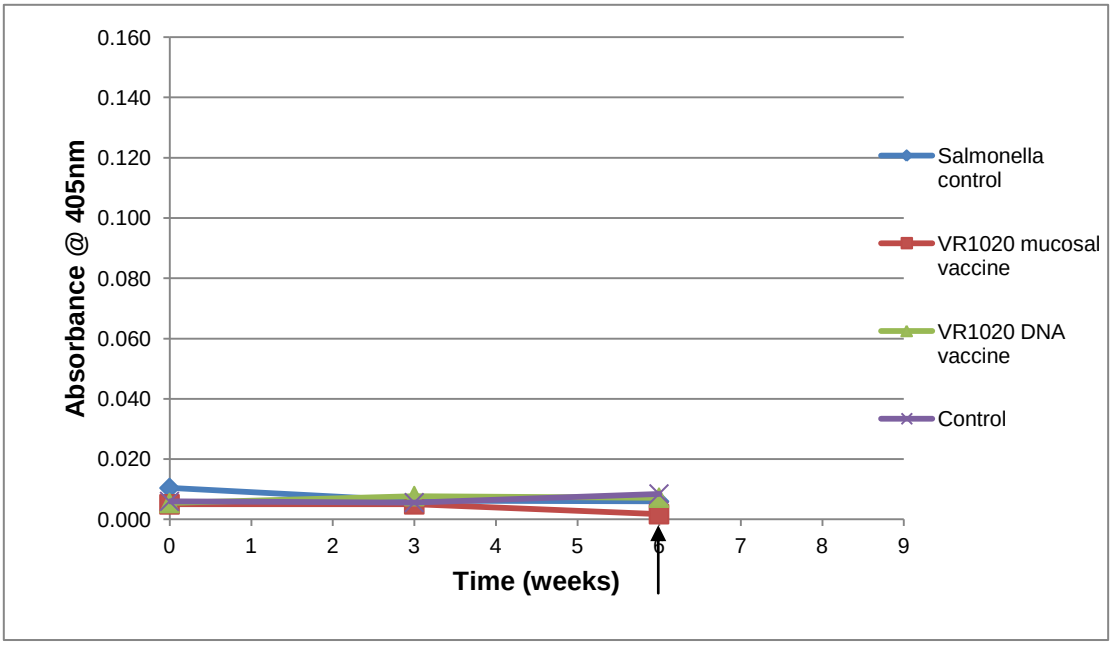

Figure 4.10: Averages of the mucosal immune responses elicited in each vaccine group as determined by an ELISA using 1 µg/ml OppA for coating, saliva collected and rabbit anti-ostrich IgA protein 2 as the detector antibody. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ VR1020 mucosal DNA vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the VR1020 naked DNA vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

Table 4.8: Statistical analysis of weight measured during the vaccine trial

Source	df	SS	MS	Pr>F
Total	359	17242.88		
Treatment	3	1543.189	514.396	0.0000
Time	2	410.744	205.372	0.0096
Treatment x Time interaction	6	101.732	16.955	0.8663
Residual	348	15187.21	43.641	

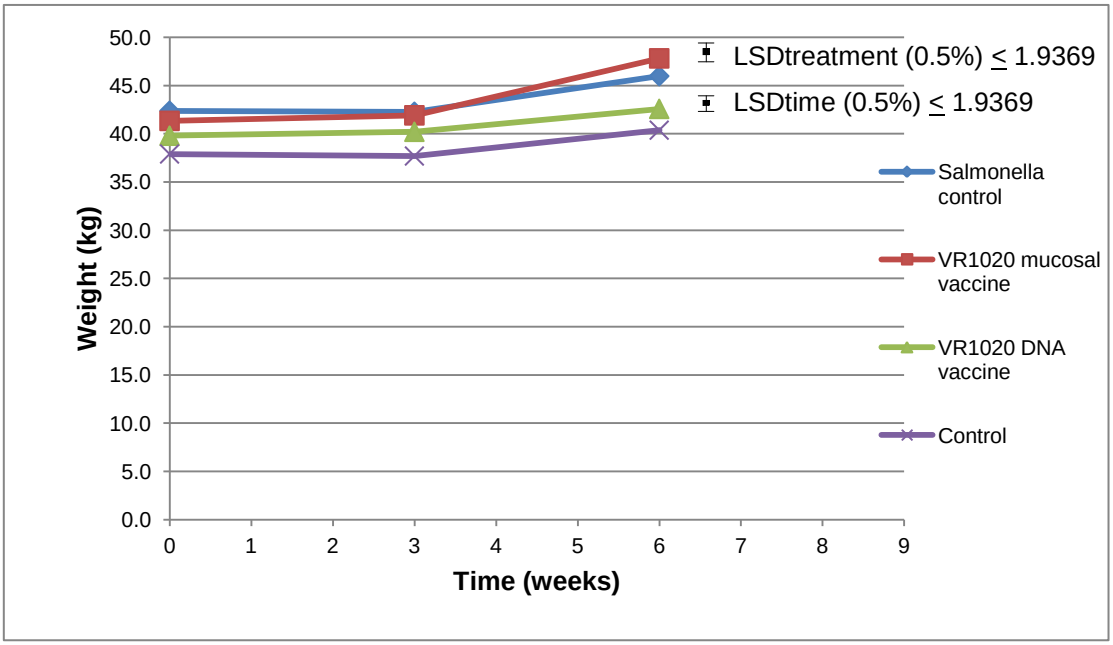

Figure 4.11: Averages of the weights measured for each vaccination group. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ VR1020 mucosal DNA vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the naked VR1020 recombinant vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

4.3.2.4. Ostrich immune response against Salmonella enterica *serovar* typhimurium *SL3261* LPS

ELISA optimisation:

The optimal coating concentration for the isolated *Salmonella enterica* serovar *typhimurium* SL3261 LPS was found to be 5.0 µg/ml. At this concentration the highest absorbance value in the ELISA of the serum sample from week 3 (2.126) was obtained and the absorbance value in the ELISA obtained with the serum sample from week 0 (1.838) remained relatively low. At a lower coating concentration, the week 3 values were too low and at a higher coating concentration, the week 0 absorbance values in the ELISA were too high. Results not shown.

Humoral and mucosal immune responses of the ostriches to *Salmonella enterica* serovar *typhimurium* SL3261:

The humoral and mucosal immune responses expressed as absorbance values obtained from the ELISA assessment of the serum and saliva samples were analysed by ANOVA and the results are shown in tables 4.9, 4.10 and 4.11. LSD of the statistically sigificant relationships are shown in the graphs of the averages of the humoral and mucosal immune response of the ostriches to *Salmonella enterica* serovar *typhimurium* SL3261. The humoral immune response against *Salmonella enterica* serovar *typhimurium* SL3261 (figure 4.12) shows a decreasing trend in all of the groups and that the response measured of the control groups was higher than the vaccinated groups. The mucosal immune response measured using the secondary ostrich IgA protein 1 and 2, figure 4.13 and 4.14, respectively, show that no mucosal immune response was observed (figure 4.13 and 4.14). The raw data and a graphical presentation of the immune responses and weights of each bird are shown in Appendix B.

As in the analyses of the humoral and mucosal immune responses against OppA, the ANOVA analysis of the humoral and mucosal immune responses against *Salmonella enterica* serovar *typhimurium* SL3261 LPS gave too low R squared values and too high C.V. values. Thus deductions that the administration of the vaccines, the *Salmonella* control treatment or the control treatment caused the observed changes in humoral or mucosal immunity could not be made and must be treated with caution. Reasons for this may be the same as those listed previously.

Table 4.9: Statistical analysis of humoral immune response measured against *Salmonella enterica* serovar *typhimurium* SL3261

Source	df	SS	MS	Pr>F
Total	359	243.951		
Treatment	3	10.061	3.354	0.0011
Time	2	19.620	9.810	0.0000
Treatment x Time interaction	6	1.374	0.229	0.8951
Residual	348	212.895	0.612	

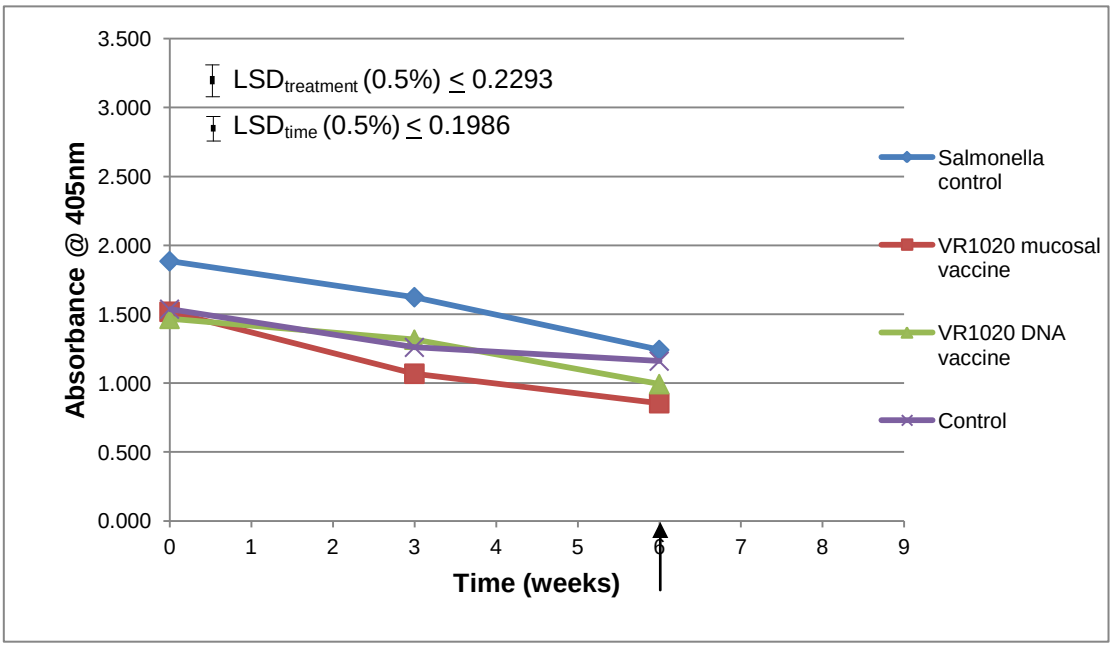

Figure 4.12: Averages of the humoral immune responses elicited in each vaccine group as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and serum collected. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ VR1020 mucosal DNA vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the VR1020 naked DNA vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

Table 4.10: Statistical analysis of mucosal immune response measured against the *Salmonella enterica* serovar *typhimurium* SL3261 using IgA protein 1 as the secondary antibody

Source	df	SS	MS	Pr>F
Total	359	0.068		
Treatment	3	0.003	0.001	0.0006
Time	2	0.001	0.000	0.2275
Treatment x Time interaction	6	0.001	0.000	0.7814
Residual	348	0.063	0.000	

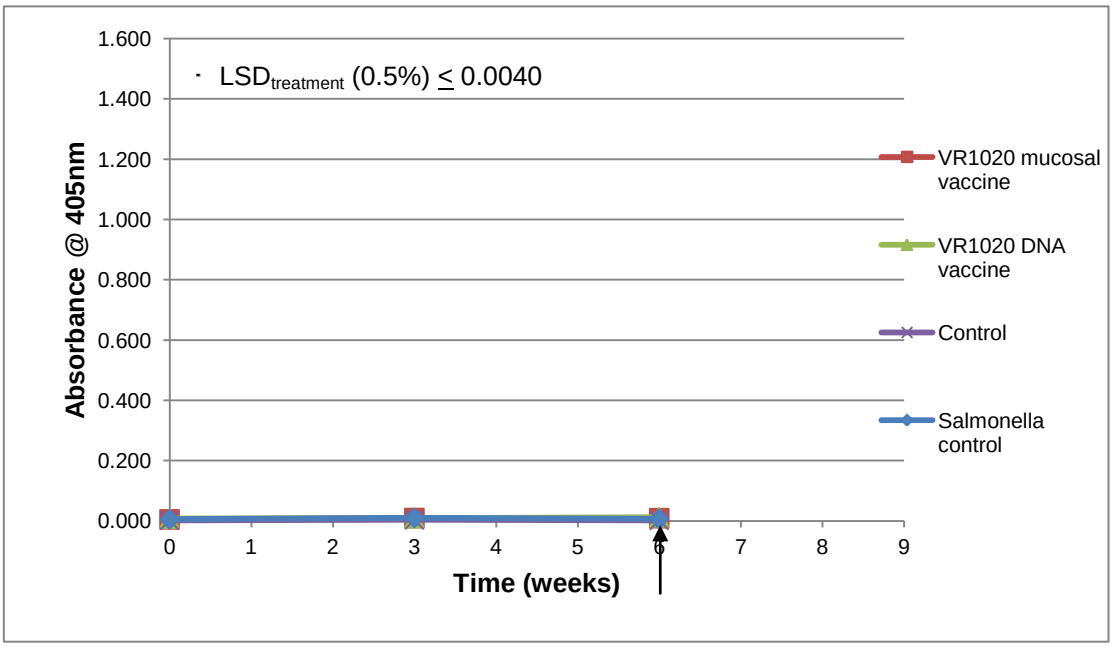

Figure 4.13: Averages of the mucosal immune responses elicited in each vaccine group as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating, saliva collected and biotinylated rabbit anti-ostrich IgA protein 1 as the biotinylated detector antibody. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ VR1020 mucosal DNA vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the VR1020 naked DNA vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

Table 4.11: Statistical analysis of mucosal immune response measured against *Salmonella enterica* serovar *typhimurium* SL3261 using IgA protein 2 as the secondary antibody

Source	df	SS	MS	Pr>F
Total	359	0.114		
Treatment	3	0.005	0.002	0.0008
Time	2	0.001	0.000	0.4364
Treatment x Time interaction	6	0.002	0.000	0.5118
Residual	348	0.107	0.000	

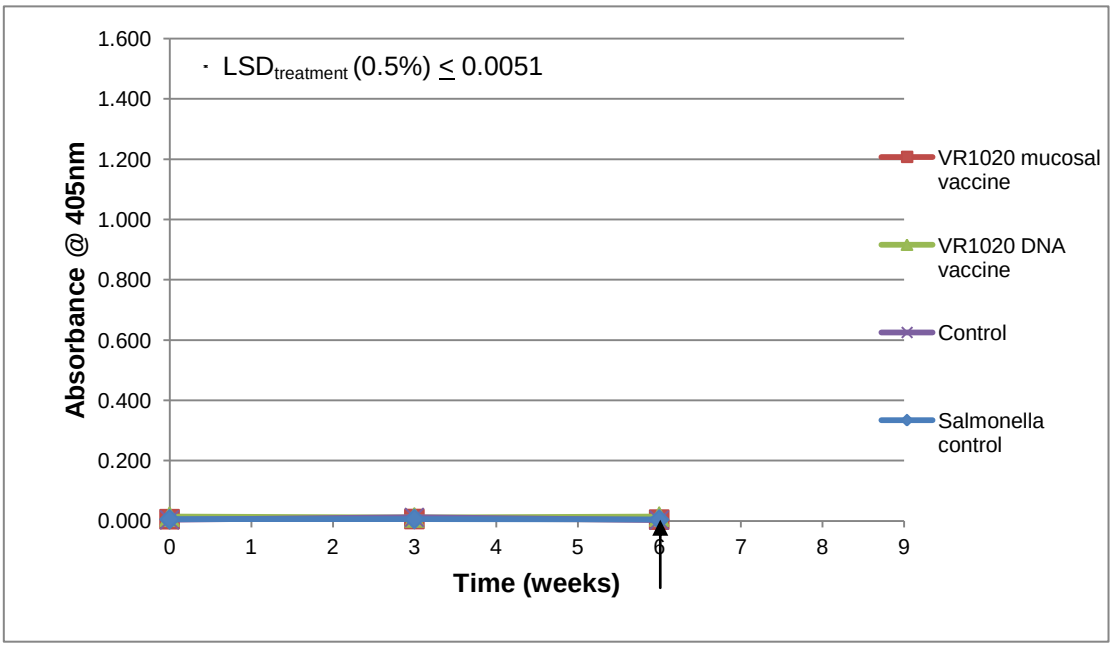

Figure 4.14: Averages of the mucosal immune responses elicited in each vaccine group as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating, saliva collected and biotinylated rabbit anti-ostrich IgA protein 2 as the biotinylated detector antibody. Group 1 (♦ *Salmonella* control) received 1 ml of a 10^9 c.f.u./ml solution of *Salmonella enterica* serovar *typhimurium* SL3261. Group 2 (■ VR1020 mucosal DNA vaccine) received 1 ml of 10^9 c.f.u./ml solution of the VR1020 mucosal DNA vaccine. Group 3 (▲ VR1020 naked DNA vaccine) received 1 ml of a 100 µg/ml solution of the VR1020 naked DNA vaccine. Group 4 (X Control) received no immunizations. The ostriches were immunized on week 0 and received a booster immunization on week 6.

4.4. Discussion

In general, it can be deduced from the results obtained from the controls used in this study, that the ELISA development for the assessment of the humoral and mucosal immune responses in ostriches against *M. struthionis* OppA and *Salmonella enterica* serovar *typhimurium* SL3261 LPS was successful.

After expression of the cloned *M. struthionis oppA* gene in *E. coli*, the GST OppA fusion protein could be isolated from the bacterial lysate using a glutathione agarose gel. After the OppA sample was cleared of impurities, reduced glutathione was used to elute the GST-fused protein without denaturation, preserving the antigenicity and function of the protein. The 130 kDa GST-OppA protein was used as a coating antigen in an ELISA to assess the immune response to OppA in an

immunized rabbit. This indicated that the antigen is suitable for use in ELISAs to analyse the humoral and mucosal immune response of the ostriches against *Mycoplasma struthionis* OppA vaccines. Similarly, it was found that LPS could be isolated from *Salmonella enterica* serovar *typhimurium* SL3261. This isolated LPS could then beused as a coating antigen in ELISAs for the detection of humoral and mucosal immune responses to *Salmonella enterica* serovar *typhimurium* SL3261 and other Salmonella LPS' as deduced from the high humoral antibody levels measured in some of the ostriches that were used in the preliminary vaccine trial.

In the saliva samples tested for *Salmonella* sp. type and ostrich mycoplasmas on weeks 0 and 3 an overwhelming percentage of the ostriches showed the presence of one or more *Salmonella* sp. type, some *Ms02* and *M. nasistruthionis* infections, but no *M. struthionis* infections. The reduction in *Salmonella* infection in week 6 can be attributed to the Tenaline® LA the ostriches received a week before the samples were collected. Tenaline® LA of which oxytetracycline is the active ingredient and reported to be active for a week after immunization (Adriaan Olivier, Personal communication), would have killed off the *Salmonella* present in these birds. After this treatment, the saliva samples still showed some Ms02 and *M. nasistruthionis*. It was surprising to find such a high incidence of *Salmonella* infections in these ostriches, although the frequent occurrence of ostrich mycoplasma infections was revealed previously in the study of Botes (2005a). This showed that most of the ostriches that were used in the vaccination trial, were already suffering from serious infections prior to the trial. This indicates that in future trials it may be beneficial to administer Tenaline® LA to ostriches two weeks before a vaccine trial is initiated to ensure that the ostriches are cleared of *Salmonella* infection.

The ANOVA analysis of the humoral and mucosal immune responses against *M struthionis* OppA and *Salmonella enterica* serovar *typhimurium* SL3261 LPS showed that the results achieved were largely invalidated by far too high levels of variation in the data. Previous vaccine trials conducted from this laboratory against Newcastle Disease Virus (Botes *et al.,* 2000) and *Mycoplasma gallisepticum* and *Mycoplasma synoviae* bacterins (Pretorius, 2009) were successful, but in these vaccination trials, the ostriches had no previous contact with the respective antigens and therefore could not have possessed prior immunity or background levels of antibodies against the antigens. However, in this trial, most of the ostriches had previous contact with *Salmonella* sp. and/or ostrich mycoplasmas. It can therefore be deduced that this was one of the factors contributing to the background values and the variation in the data. However, the low weight increase of the birds in the vaccination trial also indicated that stress may have played a role in reducing immune responses to the vaccines. Furthermore, the vaccine trial was terminated early due to an outbreak of AI in Oudsthoorn, which could have had an effect on these results as well.

For future trials, this means that the ostriches should be tested for *Mycoplasma* and *Salmonella* infection prior to the start of the trial and that an antibiotic, such as Tenaline® LA, should be administered to clear the ostriches of *Salmonella* infections. *Mycoplasma* infection in ostriches are mostly found during the winter months and when rapid changes in temperature occur, such as during changing of seasons, it might be better to plan vaccine trials for the months when the number of *Mycoplasma* infection observed in the ostriches is lower. Other factors that can be considered to increase the immune response elicited in the ostriches include studies to determine: if the dose of each vaccine administered is optimal for the ostriches to elicit an immune response and, if the immune response against the chosen protein, OppA, is able to confer protection of the ostriches against *M. struthionis*.

Chapter 5: Dosing vaccine trial

5.1. Introduction

Attempts to develop an effective vaccine against this *Mycoplasma struthionis,* started with the isolation of the candidate gene, *oppA*, and its insertion into each of three plasmids to generate three DNA vaccines (Pretorius 2009). As *M. struthionis* infects the respiratory tract of ostriches, a mucosal immune response is required, and three mucosal vaccines were developed by inserting the DNA vaccines into the carrier bacterium, *Salmonella enterica* serovar *typhimurium* SL3261, using electroporation as described in Chapter 3. The VR1020 recombinant plasmid contains the tissue plasminogen activator (tPA) signal peptide that aids in secretion of the protein after expression (Coban *et al.* 2005). As the OppA protein of *M. struthionis* is a membrane protein it was thought that this vaccine would have a better chance of success and the immune response of the VR1020 mucosal DNA vaccine was therefore compared to the VR1020 naked DNA vaccine in a preliminary vaccine trial as described in Chapter 4. This trial was compromised by a variety of factors and prematurely terminated due to AI, but the serum and saliva samples collected were still analysed and a decision was reached that the dose chosen to immunise the ostriches with the mucosal vaccine may have been too low to elicit an immune response.

In this chapter, the two mucosal vaccines that were shown to be the most stable, the VR1012 and VR1020 mucosal vaccines, were chosen to use in another vaccine trial in which the ostriches were immunised with various doses of the mucosal vaccines in an attempt to determine the effective dose needed to elicit an immune response in ostriches. The vaccine trial was performed at Kromme Rhee (Cape institute for agricultural training) in Stellenbosch, Western Cape, in an attempt to escape the threat of AI that was wide spread in Oudtshoorn at this time. Kromme Rhee is also much closer to Stellenbosch University and samples could be collected weekly in an attempt to measure the immune response of the ostriches more accurately. Swab samples were collected to test for the presence of *Salmonella* and *Mycoplasma* infections. As the chosen carrier bacterium, *Salmonella enterica* serovar *typhimurium* SL3261 is only capable of limited growth *in vivo*, cloacal swabs were collected once to test for bacterial shedding. The humoral and mucosal immune responses elicited against *Mycoplasma struthionis* OppA protein and *Salmonella enterica* serovar *typhimurium* SL3261 LPS were evaluated using the ELISAs developed in Chapter 4. An ANOVA analysis was used to analyse the data obtained.

5.2. Materials and Methods

5.2.1. Dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape.

5.2.1.1. Vaccine trial design

A vaccine trial to determine the optimal vaccine dose for ostriches to elicit an immune response was performed on 56 ostriches at Kromme Rhee (Cape institute for agricultural training) in Stellenbosch, Western Cape. The ostriches were bought from ostrich farms in the Malmesbury area moved to Kromme Rhee farm and held at for four weeks in feeding camps prior to the start of the vaccine trial. Two weeks before the trial started the ostriches were tested for *Salmonella* spp and *Mycoplasma* spp infection and all the ostriches were injected with Tenaline® LA at a dosage of 1 ml/10 kg. Tenaline® LA, which is an antibiotic containing oxytetracycline, and was administered specifically because of the high level of infection of the ostriches used in the previous trial. Background *Salmonella* infections could be expected to influence the immune responses of the ostriches to the vaccines.

The trial was designed so that there were 11 groups of ostriches. The first set of five groups consisted of five ostriches each that received different doses of the VR1012 mucosal vaccine and second set of five groups consisted of five ostriches each that received different doses of the VR1020 mucosal vaccine. The different doses were 10^9, 10^{10}, 10^{11}, 10^{12} and 10^{13} c.f.u/ml per ostrich for each of the two vaccines. The 11^{th} group consisting of six ostriches that served as a control as they received no vaccinations. The vaccine trial was carried out over a period of 9 weeks with a vaccination at week 0 and a booster vaccination at week 6. Blood samples from ostriches were collected in 5 ml Serum sep clot activator tubes (Vacuette) using 18G x 1” needles (Vacuette); every week for the first three weeks and then every three weeks to minimize the stress caused by handling the ostriches. Two saliva samples were collected from the ostriches by swabbing the trachea with two sterile swabs (plain, Rayon tipped, sterile swabs with a plastic applicator, Copan) on weeks 0, 3, 6 and 9 and one saliva sample was collected on weeks 1 and 2. The weights of the ostriches were also determined prior to the trial, and at week 2 and week 9. Two cloacal swabs of each ostrich were also taken at week 6.

5.2.1.2. Preparation of the recombinant VR1012 and VR1020 mucosal vaccine

The mucosal vaccines were prepared as described in section 4.2.1.3. with the exception that each vaccine was prepared and diluted to 10^9, 10^{10}, 10^{11}, 10^{12} and 10^{13} c.f.u./ml less than 24 hours before vaccination in sterilized serum bottles, respectively.

5.2.2. Analysis of samples collected during dosing vaccine trial on ostriches on Kromme Rhee farm in Stellenbosch, Western Cape.

5.2.2.1. Serum and saliva samples

The serum sample and one saliva sample of each ostrich was processed as described in section 4.2.2.1. and stored at -20°C. The second saliva sample collected at weeks 0, 3, 6 and 9 were analysed for ostrich *Mycoplasma* spp and *Salmonella* spp infections as described in section 4.2.2.2. and 4.2.2.3.

5.2.2.2. Cloacal samples

The first swab was streaked directly onto a plastic Petri dishes containing XLD agar and incubated at 37°C for 24 hours. The second swab was first incubated in 2 ml Selenite enrichment broth (Merck) at 43°C for 24 hours. Using a loop the enriched sample was streaked onto a plastic Petri dish containing XLD agar and incubated at 37°C for 24 hours. The XLD and Selenite enrichment broth were prepared as per manufacturer's instructions.

5.2.3. Evaluation of immune responses elicited in dosing vaccine trial against OppA

The humoral and mucosal immune responses elicited by the VR1012 and VR1020 mucosal vaccines against OppA was measured by the ELISAs used to assess the immune response against the OppA protein of *M. struthionis* as described in section 4.2.3.4. The plate layout as well as the coating concentrations and dilutions used were the same as for the samples collected for the previous vaccine trial.

5.2.4. Evaluation of immune responses elicited by dosing vaccine trial against *Salmonella enterica* serovar *typhimurium* SL3261 LPS

The humoral and mucosal immune responses the ostriches elicited against the carrier bacteria *Salmonella enterica* serovar *typhimurium* SL3261 LPS were measured by ELISA described in section 4.2.3.5. The plate layout as well as the coating concentration and dilutions used were the same as for the samples collected during the preliminary vaccine trial. The results of antibody levels measured over time with the respective ELISAs for the two vaccines and the different doses they were administered at were analysed using an ANOVA in the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

5.3. Results

5.3.1. Analysis of samples collected during vaccine trial on Kromme Rhee farm in Stellenbosch, Western Cape

The results of the analysis of *Mycoplasma* spp and *Salmonella* spp infections in the ostriches are shown in Table 5.1. At Week 0 no *Mycoplasma* spp and *Salmonella* spp infections were found in any of the ostriches. Samples collected on week 3, 6 and 9, showed some *Mycoplasma* spp and *Salmonella* spp infections. The results of the cloacal swabs to test the excretion of *Salmonella enterica* serovar *typhimurium* SL3261 are shown in Table 5.2, followed by Table 5.3 showing a guideline to the species of microorganism found on the XLD medium after 24 hours. At week 6 it could therefore be shown that most of the ostriches had varying infections, *E. coli*, *Enterobacter* spp., *Aeromonas*, *Serratia* spp and *Hafnia* spp present in their cloacae with isolated occurrences of *Citrobacter*. The only *Salmonella* sp that could be detected was *Salmonella typhosa*.

Table 5.1: Dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape

Vaccine	Ostrich nr	Weight (kg)			Infections			
		Prior	Week 2	Week 9	Week 0	Week 3	Week 6	Week 9
VR1012 Mucosal vaccine 10^9 c.f.u./ml	2	44.6	41.8	48.0				
	10	47.6	51.5	59.0				
	12	50.4	47.0	47.8				*Mycoplasma struthionis*
	18	45.2	40.2	46.6			*Salmonella*	*Mycoplasma struthionis*
	19	47.8	36.2	-			-	-
VR1012 Mucosal vaccine 10^{10} c.f.u./ml	7	44.2	43.2	39.0				
	9	46.8	-	-		-	-	-
	11	48.4	44.4	63.0				
	13	50.0	44.4	-				-
	14	47.0	40.6	53.0				
VR1012 Mucosal vaccine 10^{11} c.f.u./ml	48	48.6	47.0	53.0			*Salmonella*	
	56	48.0	40.6	44.4				
	57	47.6	44.2	44.4				
	58	47.6	36.0	48.0			*Ms02*	
	59	46.2	36.6	-		*Salmonella*	*Ms02*	-
VR1012 Mucosal vaccine 10^{12} c.f.u./ml	44	48.2	40.0	39.2				
	45	46.6	39.8	51.5			*Ms02*	*Ms02*
	51	45.6	40.0	39.4			*Salmonella*	
	52	46.8	58.5	61.0				*Mycoplasma struthionis*
	54	51.0	42.0	38.4			*Ms02*	

VR1012 Mucosal vaccine 10^{13} c.f.u./ml	23	51.6	47.6	-		*Ms02*	-	-
	32	46.6	45.0	49.8		*Ms02*		
	35	50.4	47.8	53.0				
	37	49.8	47.0	48.4				*Ms02*
	40	47.2	41.4	61.5		*Salmonella*		
Control (Nothing injected or given orally)	3	52.4	53.0	44.8			*Ms02*	
	20	52.4	47.4	38.6				
	41	51.8	41.2	55.0				
	47	54.8	47.0	64.0			*Ms02*	
	55	55.8	50.5	48.4			*Mycoplasma nasistruthi-onis*	
	53	55.4	47.2	-		*Ms02*		-
VR1020 Mucosal vaccine 10^{9} c.f.u./ml	21	48.8	51.0	-		*Ms02*		-
	24	50.0	55.5	63.0				
	26	52.8	42.8	-			-	-
	31	47.0	35.0	-		-	-	-
	36	51.2	52.5	60.0				
VR1020 Mucosal vaccine 10^{10}	25	48.2	34.6	-		*Ms02, Mycoplasma nasistruthi-onis*	-	-
	29	46.8	45.0	59.0		*Ms02*	*Ms02, Mycoplasma nasistruthi-onis*	
	30	50.0	48.2	54.0				
	33	53.8	51.0	60.5		*Ms02*	*Mycoplasma nasistruthi-onis*	
	89	53.2	54.0	59.5			*Salmonella*	
VR1020 Mucosal vaccine 10^{11} c.f.u./ml	81	44.0	46.8	43.4			*Ms02, Mycoplasma nasistruthi-onis*	*Ms02*
	82	50.8	46.2	57.5				
	64	54.0	45.8	42.6				*Mycoplasma struthionis*
	88	50.6	52.5	53.0				
	91	50.8	37.2	52.5				
VR1020 Mucosal vaccine 10^{12} c.f.u./ml	74	55.6	49.0	57.5		*Salmonella*		
	66	48.2	42.6	62.5			*Ms02*	
	71	44.8	-	-		-	-	-
	77	49.0	39.2	39.0		*Ms02*		
	79	44.6	40.4	43.6				

VR1020 Mucosal vaccine 10^{13} c.f.u./ml	65	50.4	-	-		-	-	-
	69	50.6	40.6	52.0		*Mycoplasma struthionis*		
	70	47.6	41.4	54.0				
	76	44.0	36.2	33.0		*Mycoplasma struthionis*	*Ms02*	
	78	46.2	-	-		-	-	-

[blank] No infection detected
\- No data available as ostriches died
Salmonella Multiplex PCR tested positive for a *Salmonella* sp. infection

Table 5.2: Results of cloacal swabs on XLD agar with and without enrichment

Vaccine	Ostrich nr	Cloacal swabs (Week 6)	
		Directly on XLD agar	**Enrichment prior to XLD agar**
VR1012 Mucosal vaccine Week 0: 10^{9} c.f.u./ml Week 6: 10^{9} c.f.u./ml	2	No colonies Agar red	No colonies Agar red
	10	No colonies Agar red	Yellow opaque colonies with yellow zones Agar yellow
	12	Yellow opaque colonies with yellow zones Agar yellow	Yellow opaque colonies with yellow zones Agar yellow
	18	No colonies Agar red	No colonies Agar red
	19	-	-
VR1012 Mucosal vaccine Week 0: 10^{10} c.f.u./ml Week 6: 10^{10} c.f.u./ml	7	No colonies Agar red	No colonies Agar red
	9	-	-
	11	Yellow opaque colonies with yellow zones Agar red	No colonies Agar red
	13	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	14	Yellow translucent colonies with yellow zones and black centres Agar yellow	No colonies Agar red
VR1012 Mucosal vaccine Week 0: 10^{11} c.f.u./ml Week 6: 10^{11} c.f.u./ml	48	Yellow opaque colonies with yellow zones Agar orange	Yellow opaque colonies Agar red
	56	Yellow opaque colonies with yellow zones, some colonies yellow opaque with black centres Agar yellow	No colonies Agar red
	57	Yellow opaque colonies with yellow zones Agar orange	No colonies Agar red
	58	Yellow opaque colonies with yellow zones Agar yellow	Yellow opaque colonies Agar orange
	59	Yellow translucent colonies with yellow zones and black centres Agar yellow	No colonies Agar red

VR1012 Mucosal vaccine Week 0: 10^{12} c.f.u./ml Week 6: 10^{12} c.f.u./ml	44	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	45	No colonies Agar red	No colonies Agar red
	51	Yellow opaque colonies with yellow zones Agar red	No colonies Agar red
	52	Yellow opaque colonies with yellow zones Agar yellow	Orange opaque colonies Agar yellow
	54	Yellow opaque colonies with yellow zones Agar yellow	Orange opaque colonies Agar yellow
VR1012 Mucosal vaccine Week 0: 10^{13} c.f.u./ml Week 6: 10^{13} c.f.u./ml	23	-	-
	32	No colonies Agar red	No colonies Agar red
	35	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	37	Yellow opaque colonies with yellow zones Agar orange	No colonies Agar red
	40	No colonies Agar red	No colonies Agar red
Control (Nothing injected or given orally)	3	Yellow opaque colonies with yellow zones, some colonies yellow opaque with black centres Agar yellow	No colonies Agar red
	20	Yellow opaque colonies with yellow zones Agar yellow	Yellow opaque colonies with yellow zones Agar yellow
	41	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	47	No colonies Agar red	No colonies Agar red
	55	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	53	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
VR1020 Mucosal vaccine Week 0: 10^{9} c.f.u./ml Week 6: 10^{9} c.f.u./ml	21	No colonies Agar red	No colonies Agar red
	24	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	26	-	-
	31	-	-
	36	Yellow opaque colonies with yellow zones Agar red	No colonies Agar red

VR1020 Mucosal vaccine Week 0: 10^{10} c.f.u./ml Week 6: 10^{10} c.f.u./ml	25	-	-
	29	Yellow opaque colonies with yellow zones, some colonies yellow, opaque with black centres Agar yellow	No colonies Agar red
	30	Yellow opaque colonies with yellow zones Agar red	Yellow opaque colonies with yellow zones Agar yellow
	33	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	89	No colonies Agar red	No colonies Agar red
VR1020 Mucosal vaccine Week 0: 10^{11} c.f.u./ml Week 6: 10^{11} c.f.u./ml	81	No colonies Agar red	Yellow opaque colonies with yellow zones Agar yellow
	82	Yellow opaque colonies with yellow zones Agar red	No colonies Agar red
	64	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	88	Yellow opaque colonies with yellow zones Agar orange	Yellow opaque colonies with yellow zones Agar yellow
	91	Yellow opaque colonies with yellow zones Agar orange	No colonies Agar red
VR1020 Mucosal vaccine Week 0: 10^{12} c.f.u./ml Week 6: 10^{12} c.f.u./ml	74	Yellow opaque colonies with yellow zones Agar orange	No colonies Agar red
	66	Yellow opaque colonies with yellow zones Agar yellow	No colonies Agar red
	71	-	-
	77	No colonies Agar red	No colonies Agar red
	79	Yellow opaque colonies with yellow zones Agar yellow	Orange opaque colonies Agar yellow
VR1020 Mucosal vaccine Week 0: 10^{13} c.f.u./ml Week 6: 10^{13} c.f.u./ml	65	-	-
	69	No colonies Agar red	No colonies Agar red
	70	Yellow opaque colonies with yellow zones Agar red	No colonies Agar red
	76	No colonies Agar red	No colonies Agar red
	78	-	-

- No data available as ostriches died

Table 5.3: Guidelines from Merck for identification of microorganisms on selective XLD medium

Colony appearance	Microorganism
Yellow surrounded by yellow zones, Opaque with precipitation zones	*E. coli, Enterobacter* spp, *Aeromonas* spp
Yellow surrounded by yellow zones, Opaque and mucoid with precipitation zones	*Klebsiella* spp
Yellow surrounded by yellow zones, Sometimes opaque with a black centre	*Citrobacter* spp
Yellow opaque colonies with yellow zones,	*Serratia* spp, *Hafnia* spp
Yellow surrounded by yellow zones, Translucent with a black centre	*Proteus vulgaris*
Same colour as medium (red) Translucent with black centre	*Salmonella* spp
Same colour as medium (red) Translucent	*Shigella* spp, *Providencia* spp, *Pseudomonas* spp
Orange Slightly opaque	*Salmonella typhosa*

5.3.2. Evaluation of immune responses elicited in dosing vaccine trial elicited against OppA

Humoral and mucosal immune responses of the ostriches:

The results of the immune response of the ostriches that was measured against *M. struthionis* OppA were compiled and analysed using an ANOVA (Table 5.4, 5.5 and 5.6). The results of the ANOVA analysis of the weight data of the vaccinated ostriches are shown in Table 5.7. The respective LSD values are indicated on the graphs showing the average immune response against the various doses of the VR1012 and VR1020 mucosal vaccines over time, shown in figures 5.1 to 5.6.

In the ANOVA analysis of the humoral immune response of the dosing trial, the R squared value was much higher in comparison to the R squared values obtained in the ANOVA analysis of the humoral immune response results of the preliminary vaccine trial described in Chapter 4. Although the C. V. was also much lower (54.6%) it was still not 15% or lower, meaning that the differences in humoral immune responses seen between the different vaccine doses are not statistically significant. The results achieved can therefore only be viewed as trends. The trend seen was therefore that none of the doses of the VR1012 mucosal vaccine appeared to give an immune response. The groups that received a 10^{11} c.f.u./ml dose and 10^{12} c.f.u./ml dose of the VR1020 mucosal vaccine tended to show a higher humoral response to OppA than all of the other groups including the control group.

In the ANOVA analysis of the mucosal immune response of the dosing trial, the R squared value was far too low and the C. V. much too high invalidating the data. There was no mucosal immune response measured to OppA. In general, the mucosal immune responses measured were extremely low which would also have had a negative effect on the statistical analysis. The mucosal vaccine responses against the mucosal VR1012 and VR1020 DNA vaccines using anti-ostrich IgA protein 1, are shown in figure 5.3 and 5.4, respectively, and using anti-ostrich IgA protein 2 are shown in figure 5.5 and 5.6, respectively.

Table 5.4: Statistical analysis of humoral immune response elicited against different doses of the mucosal VR1012 and VR1020 DNA vaccines against the OppA protein using anti-ostrich immunoglobulin as detection antibody

Source	df	SS	MS	Pr>F
Total	329	80.023		
Treatment	10	24.964	2.496	0.0000
Time	5	7.998	1.600	0.0000
Treatment x Time interaction	50	7.607	0.152	0.4476
Residual	264	39.454	0.149	

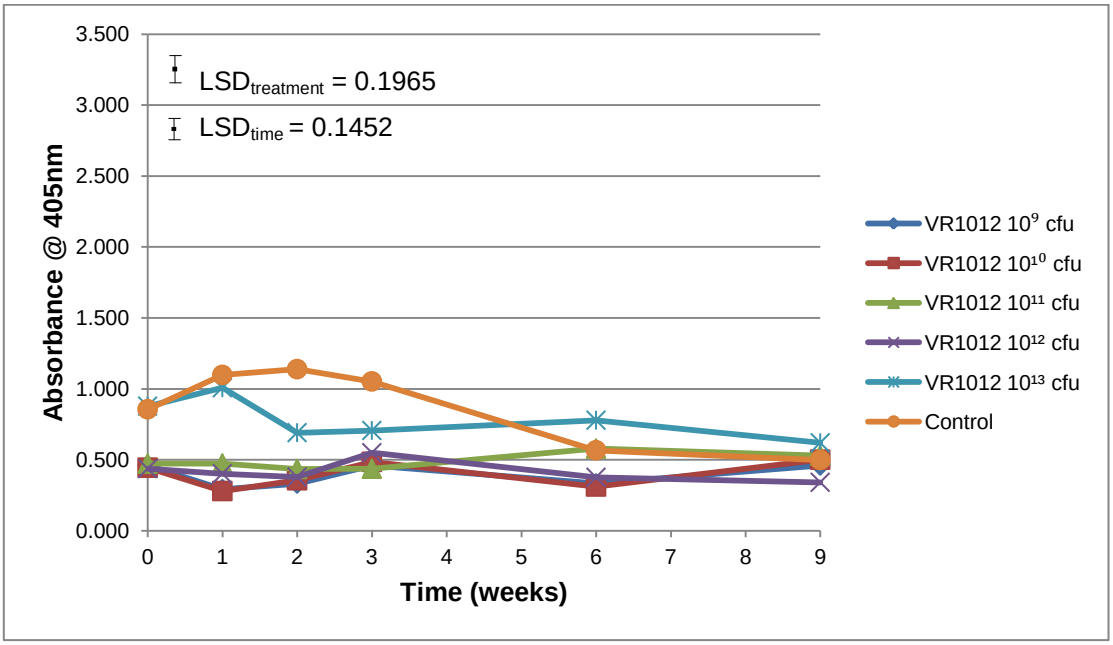

Figure 5.1: Averages of the humoral immune responses elicited by various doses of the mucosal VR1012 vaccine groups as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich Ig as detection antibody. Group 1 (♦ VR1012 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1012 mucosal vaccine, group 2 (■ VR1012 10^{10} cfu) a 10^{10} c.f.u./ml, group 3 (▲VR1012 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1012 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (✻ VR1012 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

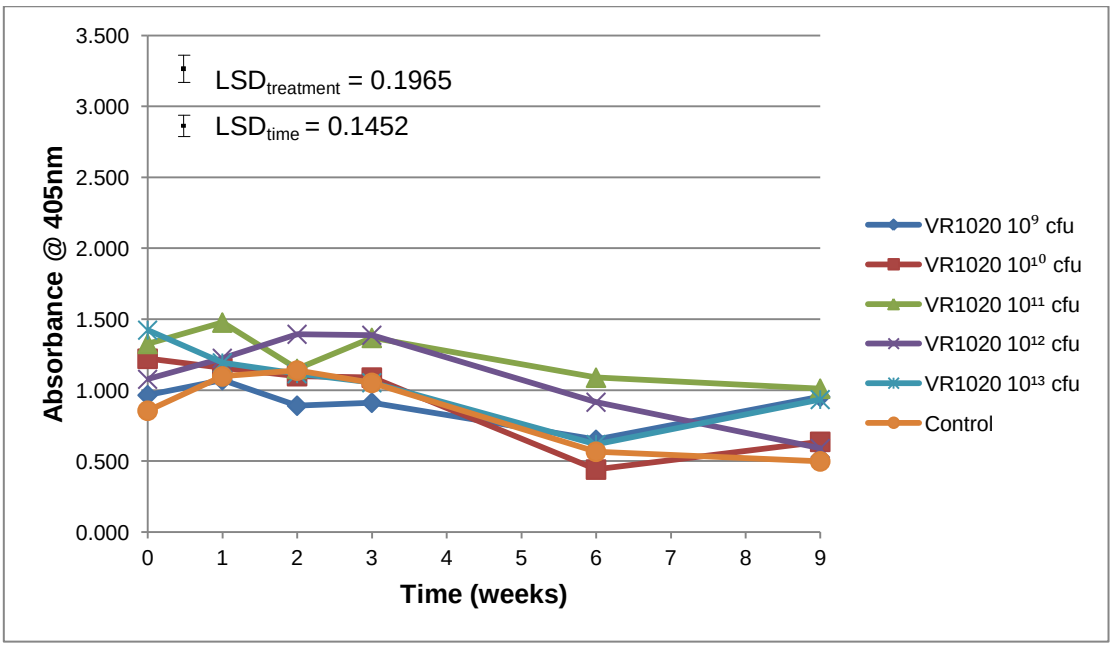

Figure 5.2: Averages of the humoral immune responses elicited by various doses of the mucosal VR1020 vaccine groups as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich immunoglobulin as detection antibody. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

Table 5.5: Statistical analysis of mucosal immune response elicited against different doses of the mucosal VR1012 and VR1020 DNA vaccines using the OppA protein using anti-ostrich IgA protein 1 as detection antibody

Source	df	SS	MS	Pr>F
Total	329	0.021		
Treatment	10	0.002	0.00	0.0023
Time	5	0.000	0.00	0.8674
Treatment x Time interaction	50	0.003	0.00	0.6448
Residual	264	0.016	0.00	

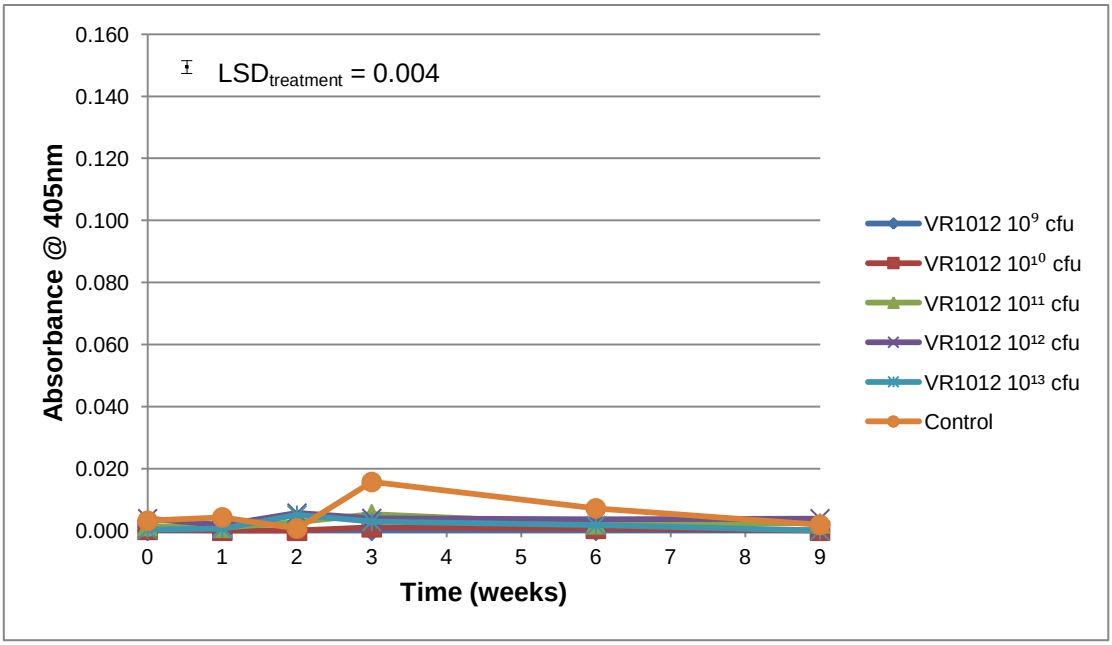

Figure 5.3: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1012 vaccine groups as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich IgA protein 1 as detection antibody. Group 1 (♦ VR1012 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1012 mucosal vaccine, group 2 (■ VR1012 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1012 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1012 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1012 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

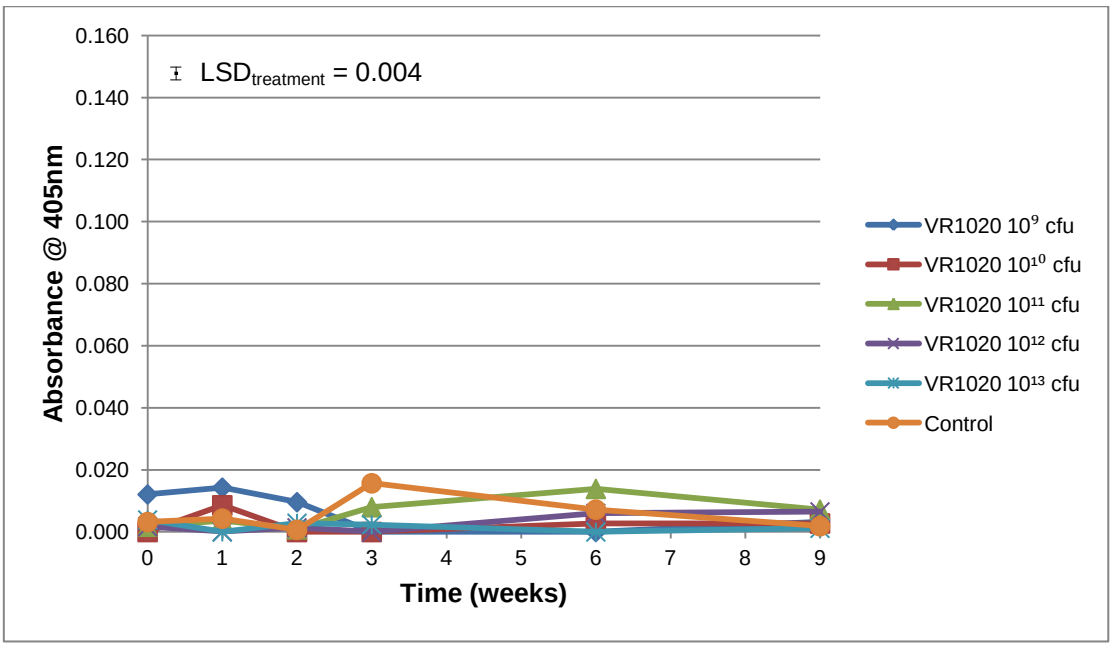

Figure 5.4: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1020 vaccine groups as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich IgA protein 1 as detection antibody. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

Table 5.6: Statistical analysis of mucosal immune response elicited against different doses of the mucosal VR1012 and VR1020 DNA vaccines using the OppA protein using anti-ostrich IgA protein 2 as detection antibody

Source	df	SS	MS	Pr>F
Total	329	0.039		
Treatment	10	0.004	0.00	0.0005
Time	5	0.001	0.00	0.4490
Treatment x Time interaction	50	0.004	0.00	0.9675
Residual	264	0.031	0.00	

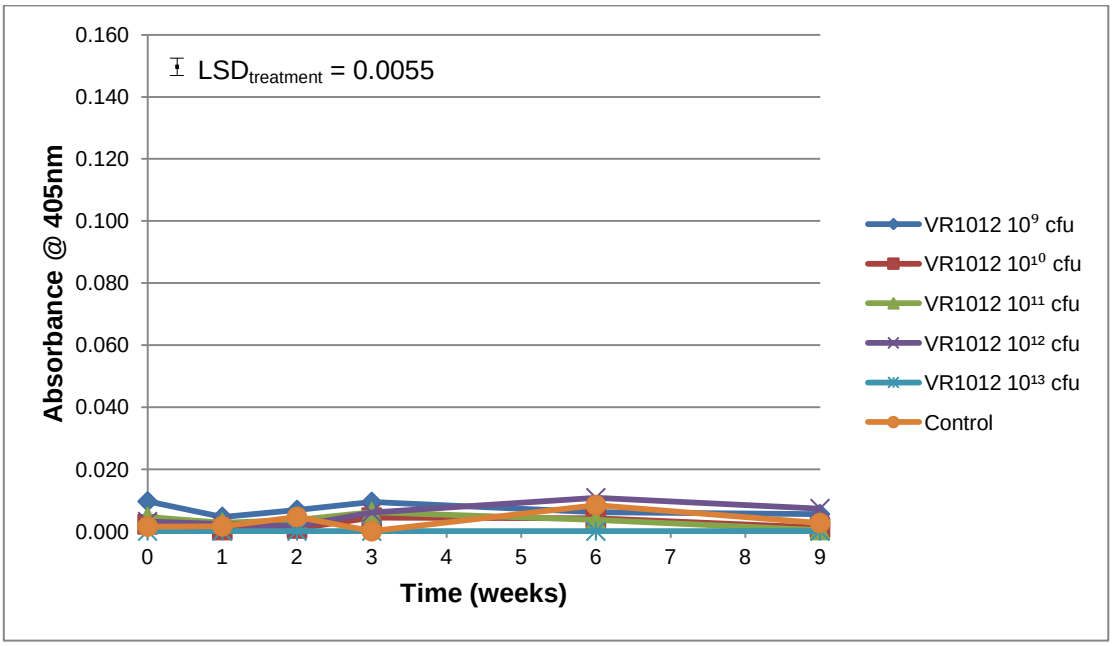

Figure 5.5: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1012 vaccine groups as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich IgA protein 2 as detection antibody. Group 1 (♦ VR1012 10^9 cfu) 10^9 c.f.u./ml solution of the VR1012 mucosal vaccine, group 2 (■ VR1012 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1012 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1012 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1012 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

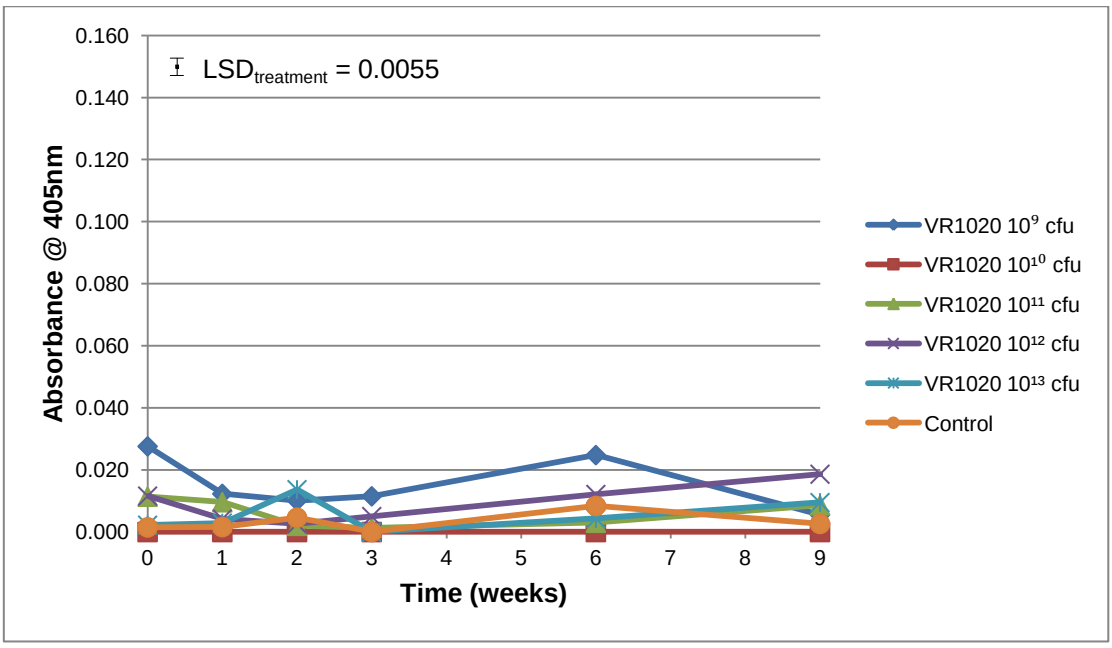

Figure 5.6: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1020 vaccine groups as determined by an ELISA using 1 µg/ml OppA for coating and anti-ostrich IgA protein 2 as detection antibody. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

Table 5.7: Statistical analysis of weight measured during the vaccine trial

Source	df	SS	MS	Pr>F
Total	329	194151.098		
Treatment	10	1971.853	197.185	0.0705
Time	5	157582.253	31516.451	0.0000
Treatment x Time interaction	50	4810.577	96.212	0.7473
Residual	264	29786.416	112.827	

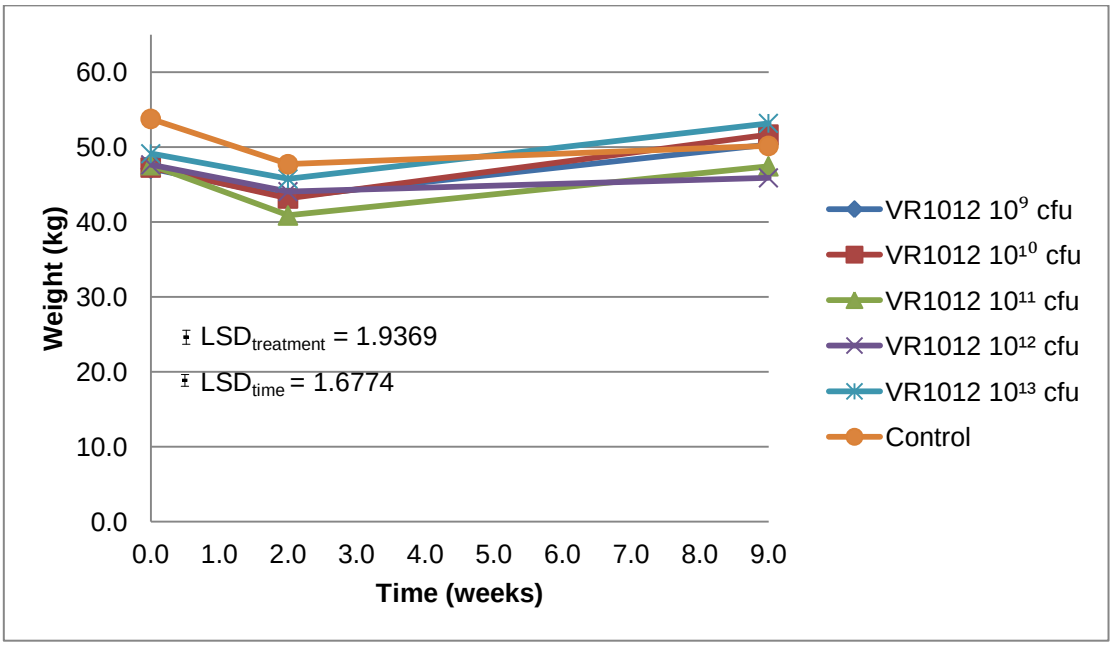

Figure 5.7: Averages of the weights measured for each vaccination group. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

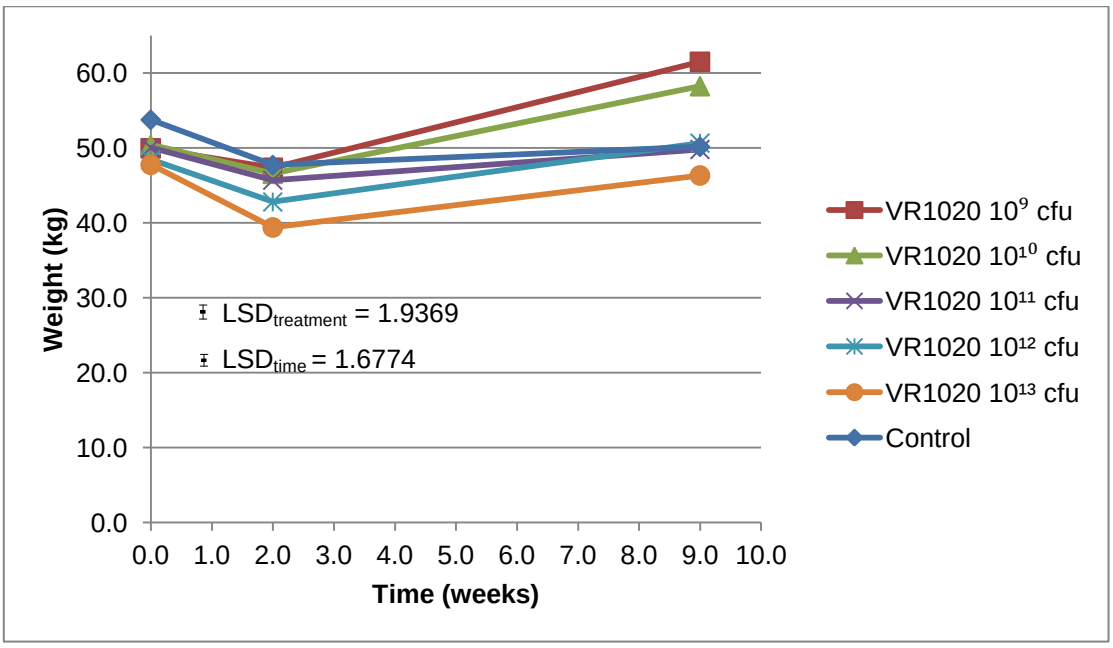

Figure 5.8: Averages of the weights measured for each vaccination group. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

5.3.3. Evaluation of immune response elicited by dosing vaccine trial against *Salmonella enterica* serovar *typhimurium* SL3261 LPS

Humoral and mucosal immune responses of the ostriches

The immune responses against the carrier bacteria were compiled, analysed using an ANOVA and the results shown in Tables 5.8, 5.9 and 5.10. The graphs of the averages of the humoral and mucosal immune responses of the ostriches are shown in figures 5.9 to 5.14. In all of the ANOVA analyses of the respective data sets too low R squared values and too high C.V. values showed that there was a very high variation in the data which does not allow deductions that the administration of the vaccines are responsible for the observed immune responses. However, a reason for this can be seen in the immune response graphs of the individual birds shown in Addendum C. In many of the groups there were isolated birds with increased antibody levels. In most cases these elevated antibody levels were seen at the start of the trial, indicating that these birds may still have retained antibodies from previous contact with *Salmonella* bacteria prior to the trial. Thus although the antibiotic treatment at the start of the trial eliminated *Salmonella* infections, the antibodies remaining from previous contact may still have been present.

Although it appears as though the group of animals that received the 10^{10} c.f.u./ml (figure 5.9) showed an increase at week 9 an examination of the titres of individual birds (figure C.10.B) show that this is the result of the increase in absorbance of only one of the ostriches in the group and is

therefore not significant. It was also observed that the humoral immune response values observed was generally higher than the mucosal immune response values.

Table 5.8: Statistical analysis of humoral immune response elicited against different doses of the mucosal VR1012 and VR1020 DNA vaccines against the *Salmonella enterica* serovar *typhimurium* SL3261 LPS using anti-ostrich immunoglobulin as detection antibody

Source	df	SS	MS	Pr>F
Total	329	146.639		
Treatment	10	30.985	3.099	0.0000
Time	5	10.501	2.100	0.0000
Treatment x Time interaction	50	20.993	0.420	0.0887
Residual	264	84.159	0.319	

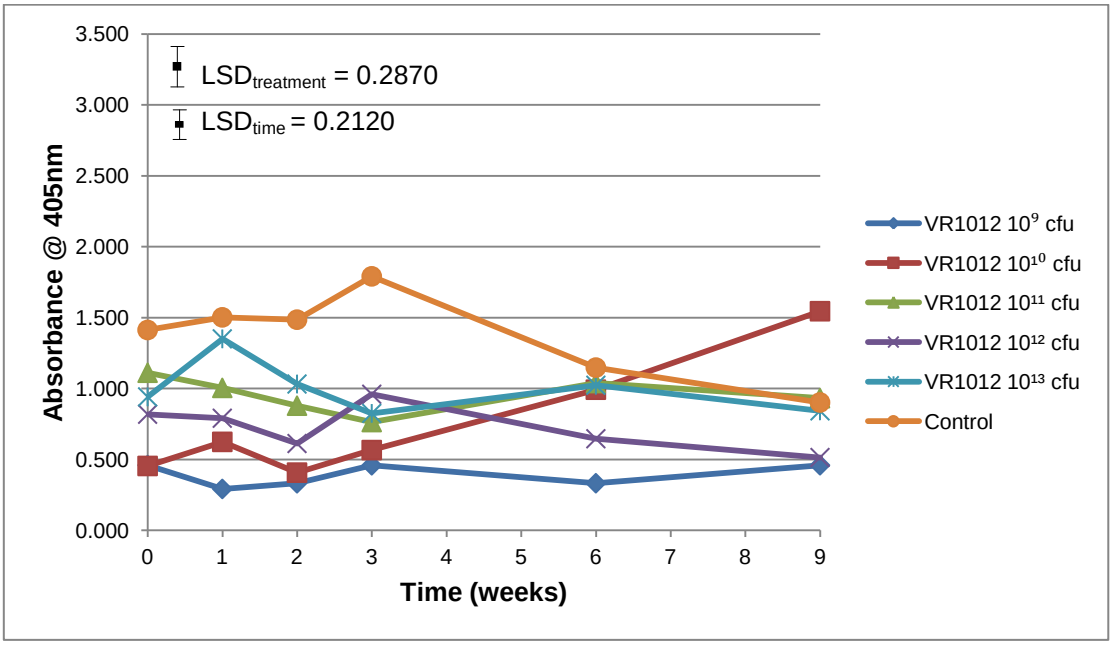

Figure 5.9: Averages of the humoral immune responses elicited by various doses of the mucosal VR1012 vaccine groups as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and anti-ostrich immunoglobulin as detection antibody. Group 1 (♦ VR1012 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1012 mucosal vaccine, goup 2 (■ VR1012 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1012 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1012 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1012 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

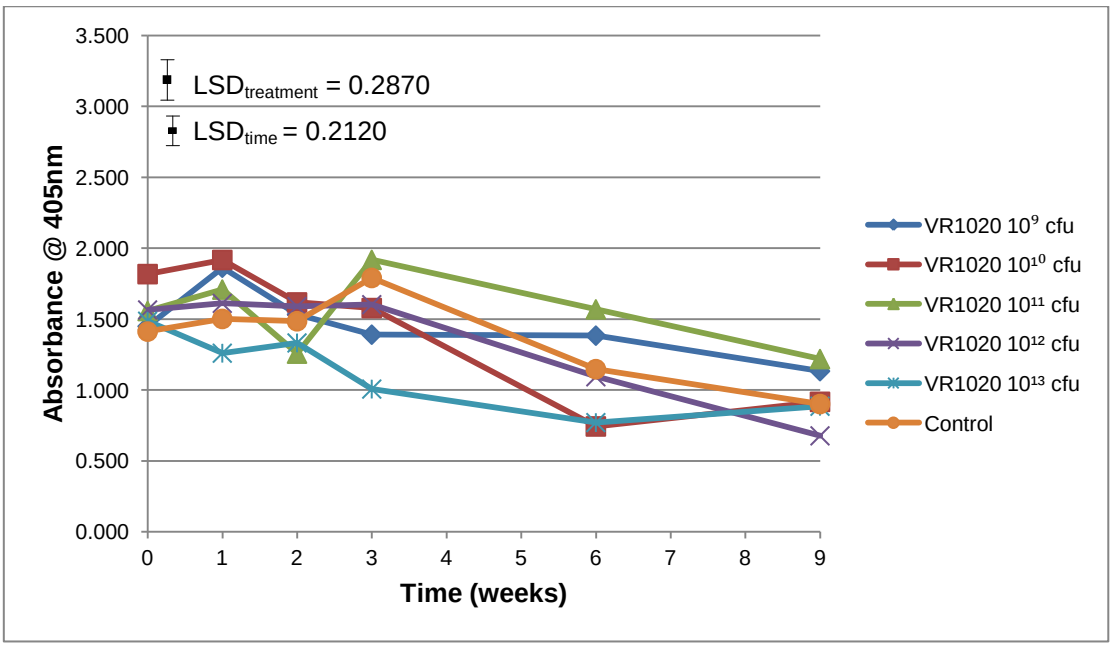

Figure 5.10: Averages of the humoral immune responses elicited by various doses of the mucosal VR1020 vaccine groups as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and anti-ostrich immunoglobulin as detection antibody. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

Table 5.9: Statistical analysis of humoral immune response elicited against different doses of the mucosal VR1012 and VR1020 DNA vaccines against the *Salmonella enterica* serovar *typhimurium* SL3261 LPS using anti-ostrich IgA protein 1 as detection antibody

Source	df	SS	MS	Pr>F
Total	329	0.034		
Treatment	10	0.004	0.000	0.0000
Time	5	0.000	0.000	0.6805
Treatment x Time interaction	50	0.003	0.000	0.9744
Residual	264	0.026	0.000	

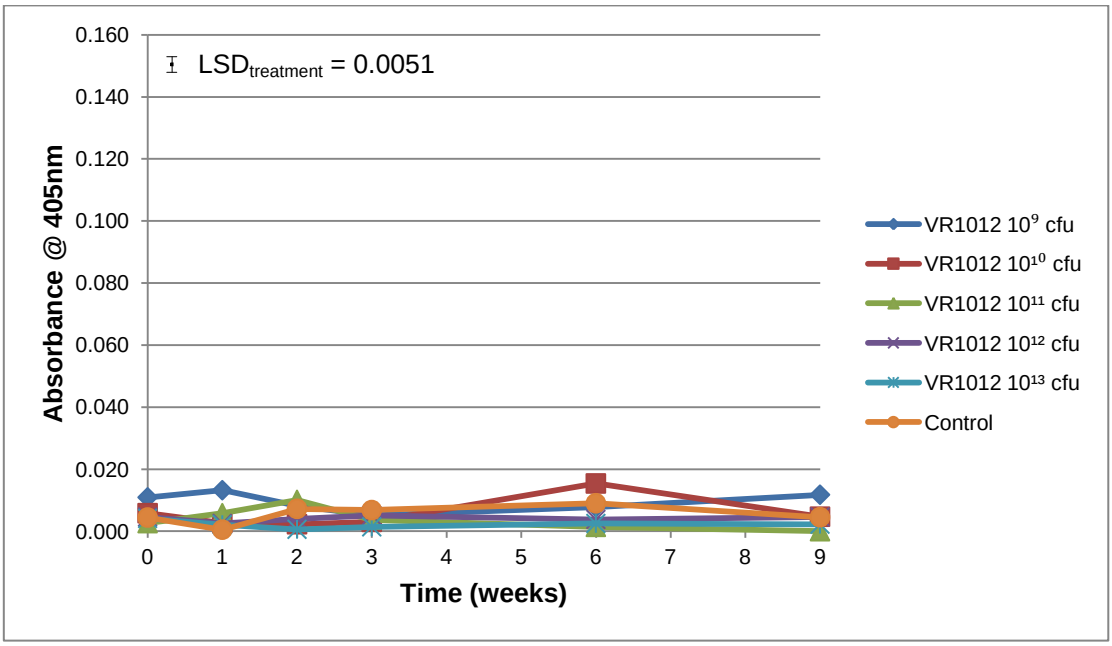

Figure 5.11: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1012 vaccine groups as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and anti-ostrich IgA protein 1 as detection antibody. Group 1 (♦ VR1012 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1012 mucosal vaccine, group 2 (■ VR1012 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1012 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1012 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1012 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

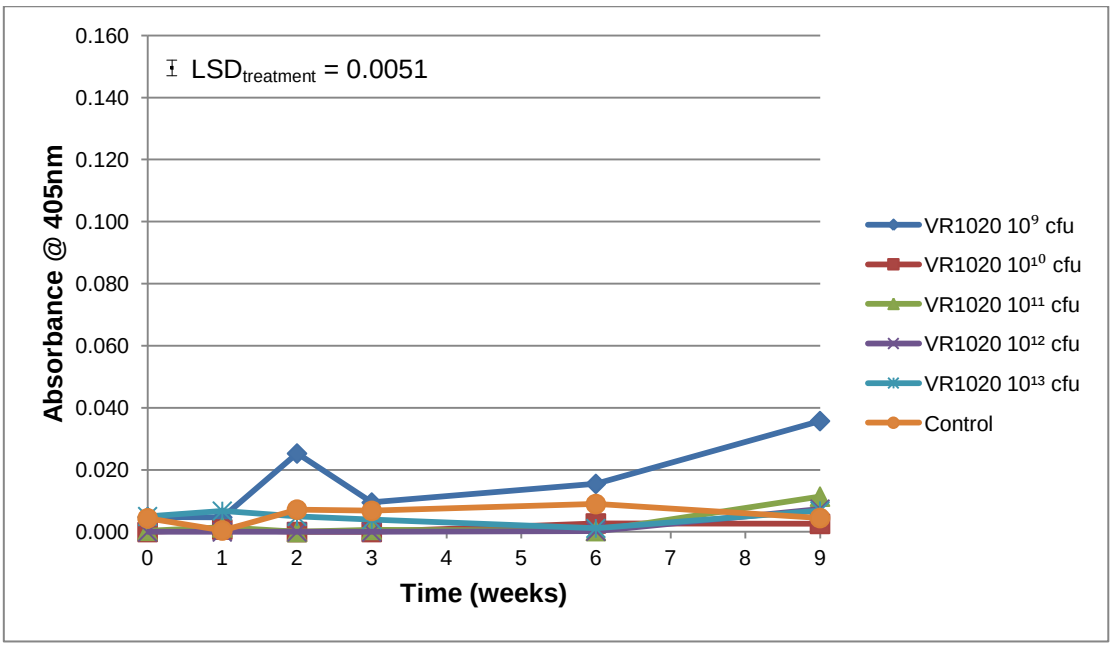

Figure 5.12: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1020 vaccine groups as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and anti-ostrich IgA protein 1 as detection antibody. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, Group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml, Group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml, Group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

Table 5.10: Statistical analysis of humoral immune response elicited against different doses of the mucosal VR1012 and VR1020 DNA vaccines against the *Salmonella enterica* serovar *typhimurium* SL3261 LPS using anti-ostrich IgA protein 2 as detection antibody

Source	df	SS	MS	Pr>F
Total	329	0.083		
Treatment	10	0.011	0.001	0.0000
Time	5	0.001	0.000	0.3746
Treatment x Time interaction	50	0.011	0.000	0.5942
Residual	264	0.060	0.000	

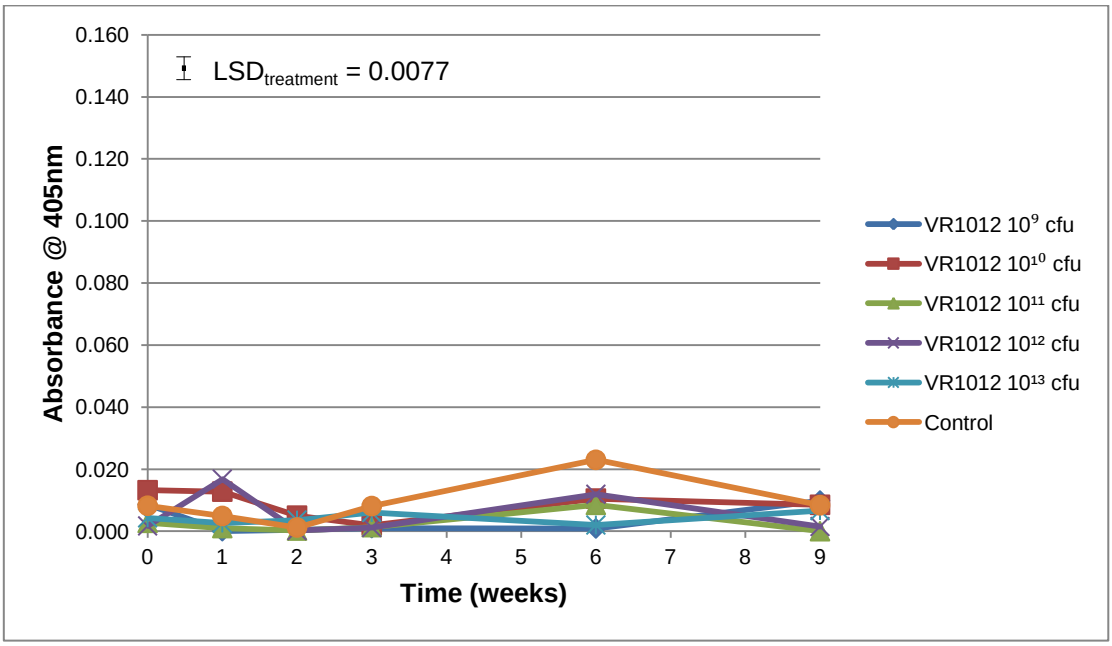

Figure 5.13: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1012 vaccine groups as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and anti-ostrich IgA protein 2 as detection antibody. Group 1 (♦ VR1012 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1012 mucosal vaccine, group 2 (■ VR1012 10^{10} cfu) 10^{10} c.f.u./ml, group 3 (▲VR1012 10^{11} cfu) 10^{11} c.f.u./ml, group 4 (X VR1012 10^{12} cfu) 10^{12} c.f.u./ml, group 5 (Ж VR1012 10^{13} cfu) 10^{13} c.f.u./ml, group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

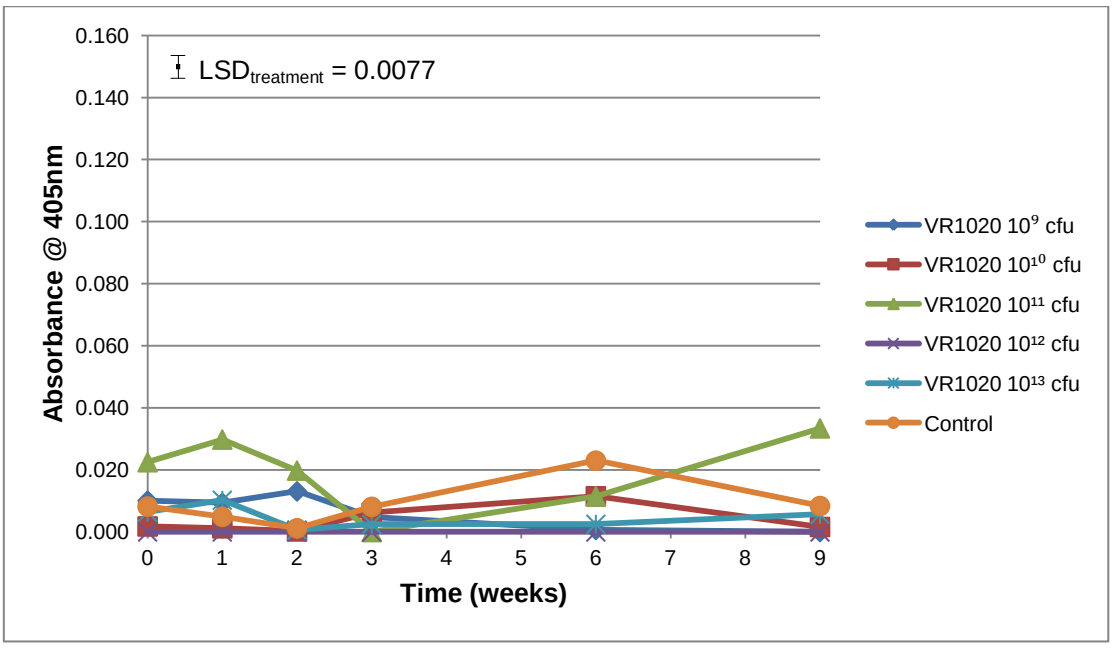

Figure 5.14: Averages of the mucosal immune responses elicited by various doses of the mucosal VR1020 vaccine groups as determined by an ELISA using 5 µg/ml *Salmonella enterica* serovar *typhimurium* SL3261 LPS for coating and anti-ostrich IgA protein 2 as detection antibody. Group 1 (♦ VR1020 10^9 cfu) received 1 ml of a 10^9 c.f.u./ml solution of the VR1020 mucosal vaccine, group 2 (■ VR1020 10^{10} cfu) 10^{10} c.f.u./ml., group 3 (▲VR1020 10^{11} cfu) 10^{11} c.f.u./ml., group 4 (X VR1020 10^{12} cfu) 10^{12} c.f.u./ml., group 5 (Ж VR1020 10^{13} cfu) 10^{13} c.f.u./ml., group 6 (● Control) received no immunizations. The ostriches were immunized at week 0 and received a booster immunization at week 6.

5.4. Discussion

Before the ability of the mucosal DNA vaccines to elicit a humoral and mucosal immune response could be evaluated further it was decided to determine the optimal dose required to elicit an immune response in ostriches. In the literature the dose mostly used was $1x10^9$ c.f.u./ml, which includes various studies on mice (Benitez, McNair & Mead 2009, Poirier, Kehoe & Beachey 1988), and chickens (Pogonka *et al.* 2003). Some of the other doses found in literature include $5x10^{10}$ c.f.u./2 ml in dogs (Chabalgoity *et al.* 2000), $1x10^{11}$ c.f.u./ml in mice (Barry *et al.* 1996), $5x10^{11}$ per cat (Tijhaar *et al.* 1997) and in rabbits doses between 10^6 and $>10^{10}$ c.f.u./rabbit was evaluated to determine a target dose of 10^8-10^9 c.f.u./rabbit. With this in mind doses chosen were between 10^9 and 10^{13} c.f.u./ml also taking into account the larger size of ostriches in comparison to chickens.

In comparison to the preliminary vaccine trial, precautions were taken to prevent making the same mistakes. The injection of the ostriches with Tenaline® LA two weeks prior to the start of the trial, appeared to have brought all bacterial infections under control at the start of the dose range vaccine trial. Tenaline® LA is reported to be active for a week after immunization (Adriaan Olivier, Personal communication). As the birds were vaccinated two weeks after the Tenaline® LA administration it could not have inactivated the *Salmonella enterica* serovar *typhimurium* SL3261 vaccine carrier, and this could not be responsible for the lack of immune response to OppA.

Three weeks after the start of the trial isolated *Salmonella* infections were found, which the ostriches probably contracted from the environment, contaminated feedlots or neighbouring untreated ostriches. However, ostrich mycoplasma infections were found indicating that the birds had either carried these pathogens or had been infected from neighbouring untreated ostriches. However, a decrease in the weight of the ostriches was observed in week 2, indicating that the ostriches were very stressed and the ostriches that showed a large decline in their weight were given liver stimulant on week 3, which is a substance that aids in increasing the general condition of the ostriches. Furthermore, 13 ostriches died during the trial, which from their general behaviour, we deduced was due to the fact that they were not used to being kept in small feedlot camps causing high levels of stress. In general, we also observed that the vaccination and sample taking was very stressful for the ostriches. Taken together, these factors all must have contributed to the high levels of variation in the immune response data, which unfortunately invalidated the trial once again.

However, a positive result of this trial was that the vaccine strain could not be detected either by direct plating or by enrichment of the cloacal swab in selenite broth before plating on XLD agar, thus the carrier bacteria could not be detected in the ostriches six weeks after immunization. This means that the chosen strain does not persist *in vivo* and will only affect the birds for a short period which is precisely what is aimed for in using these bacteria as carriers.

At the end of the trial, the controlling veterinarian actually confirmed that some of the birds that were in the trial, had been tested at random for AI, and had tested positive. Thus this trial was also adversely influenced by AI and this must have contributed to the variation in the immune response results seen in the trial. The general conclusion that can therefore be drawn from this trial is that factors contributing to background levels of immunity need to be tightly regulated before any trials using mucosal DNA vaccines can be conducted in ostriches, as will be discussed in chapter 6.

Chapter 6: Discussion and future perspectives

In this study, the three previously developed naked pCI-neo, VR1012 and VR1020 DNA vaccines were successfully electroporated into the carrier bacterial strain, *Salmonella enterica* serovar *typhimurium* SL3261, and three corresponding mucosal DNA vaccines were developed. The *in vivo* stability (figure 3.8) of the mucosal DNA vaccines showed that the VR1012 and VR1020 mucosal DNA vaccines were more stable than the pCI-neo mucosal DNA vaccines and are therefore the best candidates for future use as mucosal DNA vaccines.

Subsequently a preliminary vaccine trial using the VR1020 naked DNA vaccine and the mucosal VR1020 DNA was conducted. The VR1020 mucosal DNA vaccine was chosen due to the presence of a tPA signal peptide that aids in secretion of the expressed protein. The mucosal DNA vaccine was given orally and was viewed to be a less stressful method of administration than the intramuscular injection required for the naked DNA vaccine (Qu *et al.* 2008). The mucosal DNA vaccine is also easier and faster to produce than naked DNA vaccines (Schoen *et al.* 2004). The use of bacteria as carriers of DNA vaccines has another advantage in that it can act as a bivalent vaccine, conferring protection against the pathogen and the carrier bacteria, thus the immune responses of the ostriches were evaluated against *M. struthionis* OppA and *Salmonella enterica* serovar *typhimurium* SL3261 LPS.

The ELISAs developed were successful for measuring the immune responses elicited by the ostriches in both the immunized groups and the control groups. However, the outbreak of AI in the Oudtshoorn district in 2011 ended the preliminary vaccine trial prematurely. This together with prior exposure to *Salmonella* and *Mycoplasma* infections caused a high amount of variation observed in the immune response data, which resulted in an inconclusive trial, an immune response to Opp A could not be detected in the vaccinated ostriches. It was concluded from the trial that prior testing for *Mycoplasma* and *Salmonella* infection is needed to assess the influence of these bacteria on the analysis of the immune response of the ostriches. Also the mucosal DNA vaccine dose of 10^9 c.f.u./ml used to immunise the ostriches might not have been an effective dose to elicit an immune response.

To determine an effective dose required to elicit an immune response in ostriches a second vaccine trial was performed, this time with various doses of the mucosal VR1012 and VR1020 DNA vaccines. For this trial the antibiotic, Tenaline® LA, was administered to clear pre-existing *Salmonella* infections and aid in preventing these bacteria from influencing the immune responses of the ostriches to the DNA vaccine. The vaccine trial to determine the dose required to elicit an immune response gave results that again showed far too high levels of variation making the analysis of the statistical data for all the data sets invalid. The trend observed was that the VR1020 mucosal DNA vaccine gave better immune responses than the VR1012 mucosal DNA vaccine,

possibly due to the tPA signal peptide encoded upstream of the Opp A gene in the VR1020 DNA vaccine plasmid. Another trend observed was that the 10^{11} c.f.u./ml and 10^{12} c.f.u./ml doses of the VR1020 mucosal DNA vaccine elicited better immune responses than the other doses and might be a good starting point for future vaccine trials using the mucosal DNA vaccine in ostriches. This trial was compromised by the severe stress observed in the ostriches, which was clear from the lack of weight gain and in some instances loss of weight observed in the first three weeks of the vaccine trial. This might have impaired the ability of the ostriches to elicit an effective immune response. Thus, further vaccine trials will have to be carried out to determine if the mucosal DNA vaccines can elicit immune responses.

Even though the vaccine trials conducted in this study were not successful, the validity of the ELISAs developed could be confirmed and can be used for the analysis of future vaccine trials and a number of suggestions on how to improve the immune response elicited by ostriches could be made. In future trials steps should be taken to decrease the stress experienced by the ostriches, ostriches should not be moved prior to the start of a trial, and blood samples should not be collected and/or ostriches vaccinated at intervals less than three weeks. Furthermore the housing of ostriches in small feedlot enclosures should be avoided due to the stress that this housing causes. Another step that can be taken in the future to ensure better immune responses is to attempt vaccine trials on secluded ostrich farms, where the risk of avain influenza infection may be lower. A valuable consideration might also be to use a flock free of *Salmonella* infection. The antibodies levels present in the ostriches against both *Mycopoplasma struthionis* and *Salmonella* can be tested using the developed ELISAs prior to the start of the vaccine trial, and then the trial started only once the ELISA results are negative.

With regard to the mucosal vaccines considerations for future studies might be to first test the ability of the *Salmonella* carrier to enter cells and to release the OppA gene containing plasmid as well as the expression of the OppA protein after its release from the *Salmonella* carrier *in vitro* (Guo *et al.* 2003). Guo *et al.* (2003) mixed *Salmonella enterica* serovar *typhimurium* SL3261 strain carrying a DNA vaccine plasmid containing a enhanced green florescent gene, with murine peritoneal macrophages. The expression of the enhanced green florescent gene was then observed using flow cytrometry and it was shown that the protein was expressed when viewed after 48 hours. Grillot-Courvalin *et al.* (2002) transfected COS-1 cells with *Salmonella enterica* serovar *typhimurium* SL7207 carrying a plasmid containing the green florescent gene to show gene transfer. Similar studies to test these vaccines for use in ostriches should be considered in chicken cells which are more commonly available as tissue culture cell lines.

Another consideration would be to perform a preliminary study on smaller animals before further trials on ostriches. Chickens may be a suitable species to use in preliminary trials because of the fact that they are domesticated and will not experience the stress that ostriches apparently do, and because of the relatedness to ostriches this may produce results which could indicate that ostriches would respond similarly.

Also a heterologous prime-boost immunization strategy can be considered, where the mucosal vaccine is first used to prime the immune response of the host, followed by a booster immunization with recombinant OppA protein. The OppA protein that was expressed recombinantly for ELISA plate coating purposes in this study, could then be used as the booster protein.

Apart from all the external factors that influenced the vaccine trials, mucosal DNA vaccines were developed that have the potential to elicit an immune response in ostriches, and the strategy can be applied to the vaccine development against the other two ostrich-specific mycoplasmas, *Ms02* and *M. nasistruthionis*. The use of bacteria as carriers of DNA vaccines also has the potential to be a multivalent vaccine, conferring protection against the carrier bacteria as well as more than one pathogen (Vindurampulle & Attridge 2003). Another potential future perspective for the project might be to insert the DNA vaccines for all three ostrich-specific *Mycoplasmas* into a single *Salmonella enterica* serovar *typhimurium* SL3261 bacterium to elicit immune responses against all three ostrich mycoplasmas simultaneously which would obviously have huge practical and economic advantages.

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Addendum A: Stability of recombinant plasmid vaccines in *S. enterica* serovar *typhimurium.*

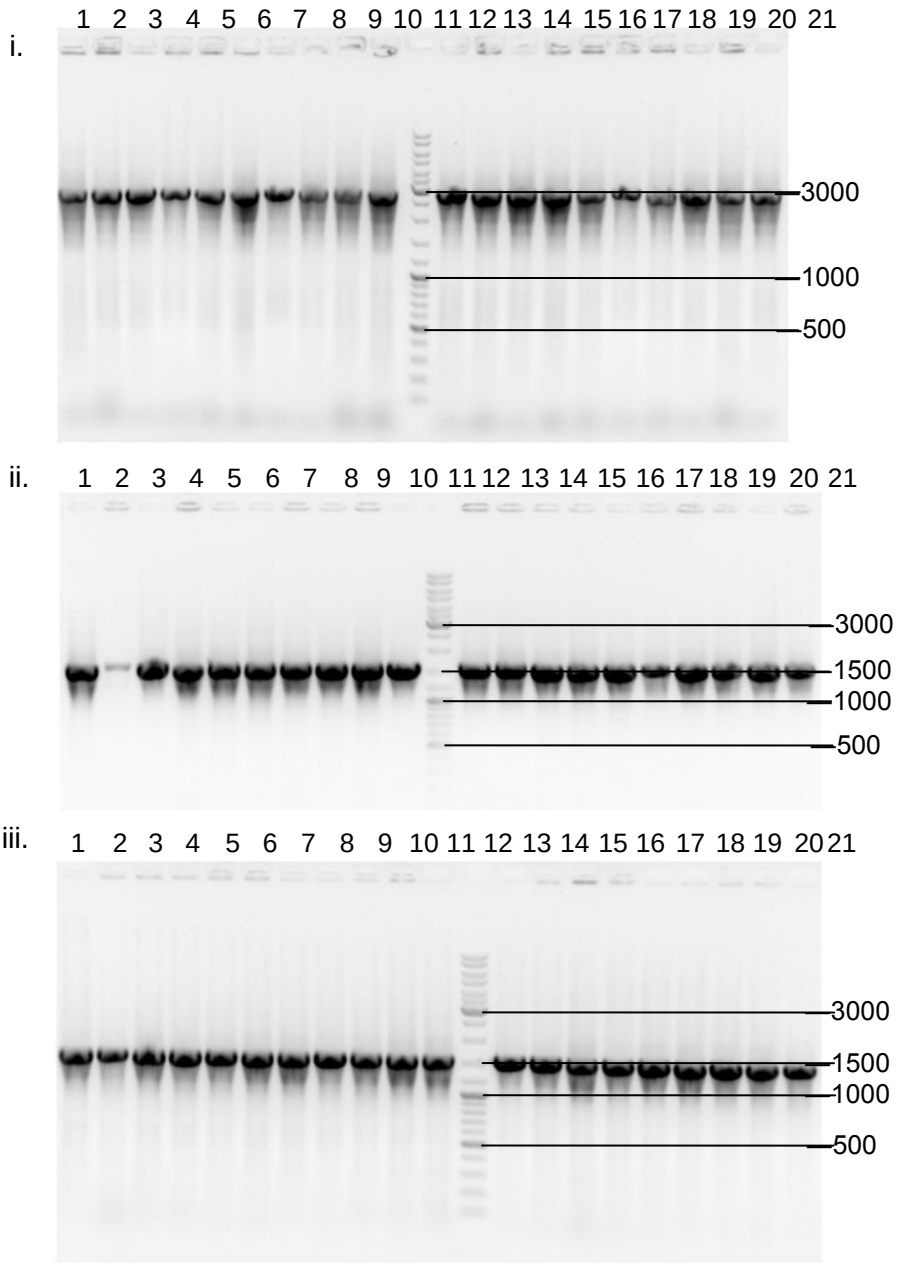

Figure A1. Day 1 of stability assessment of DNA vaccines in *Salmonella enterica* serovar *typhimurium* SL3261 tested with a diagnostic colony PCR. i. Mucosal pCIneo DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, ampicillin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, ampicillin. ii. VR1012 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin. iii. VR1020 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 12: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 11, 13-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin.

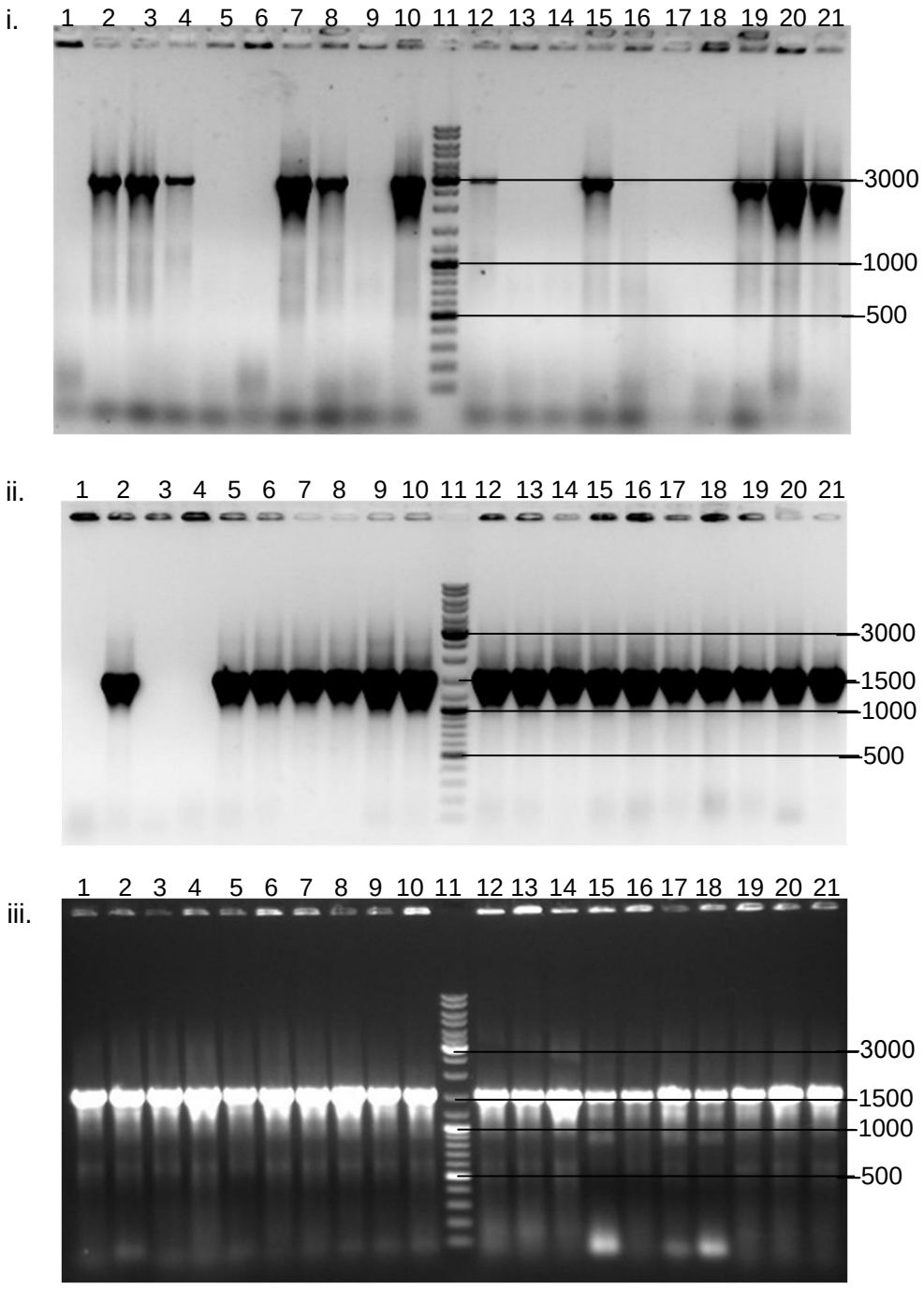

Figure A2: Day 2 of stability assessment of DNA vaccines in *Salmonella enterica* serovar *typhimurium* SL3261 tested with a diagnostic colony PCR. i. Mucosal pCIneo DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, ampicillin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, ampicillin. ii. VR1012 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin. iii. VR1020 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin.

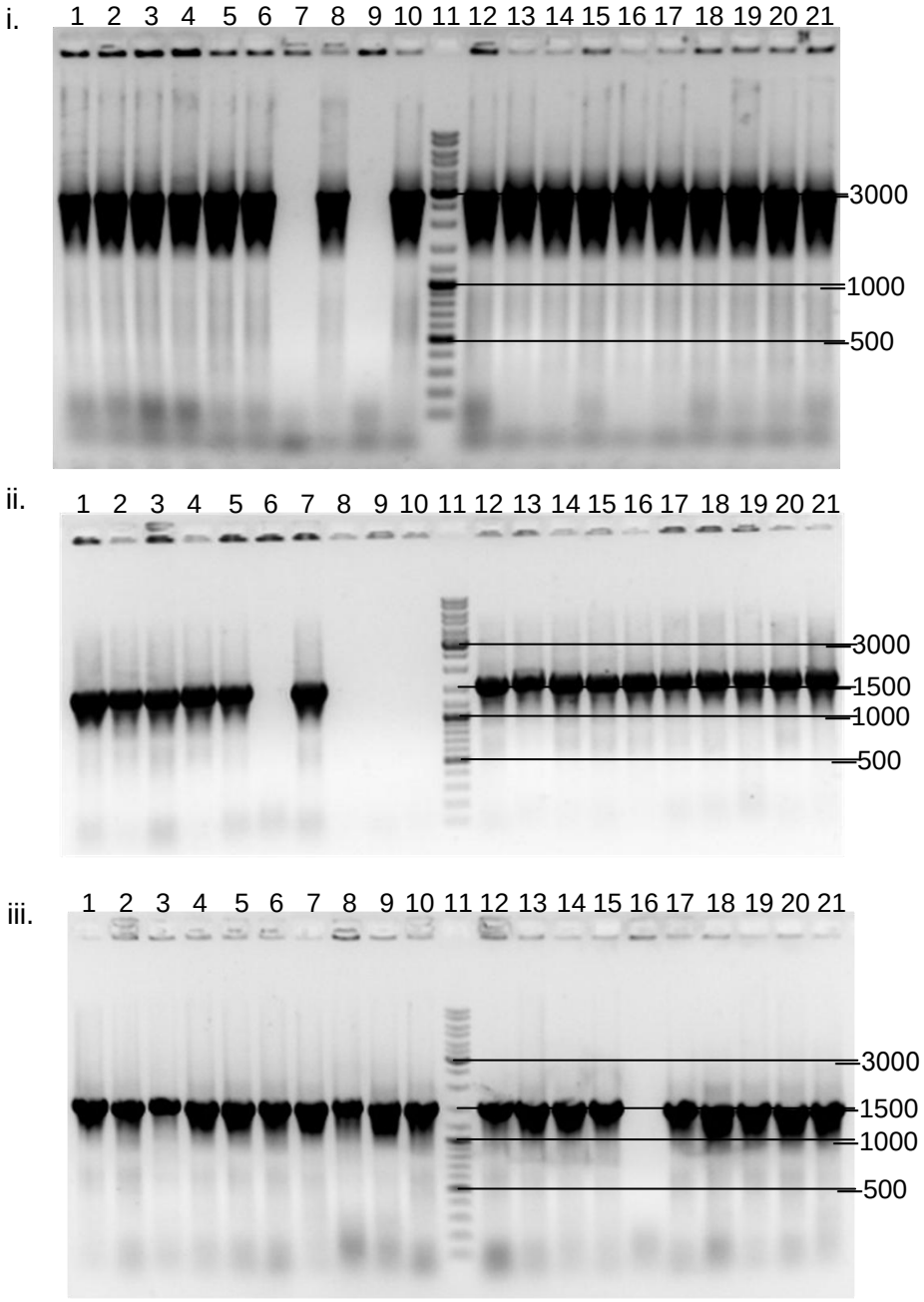

Figure A3: Day 3 of stability assessment of DNA vaccines in *Salmonella enterica* serovar *typhimurium* SL3261 tested with a diagnostic colony PCR. i. Mucosal pCI-neo DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, ampicillin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, ampicillin. ii. VR1012 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin. iii. VR1020 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin.

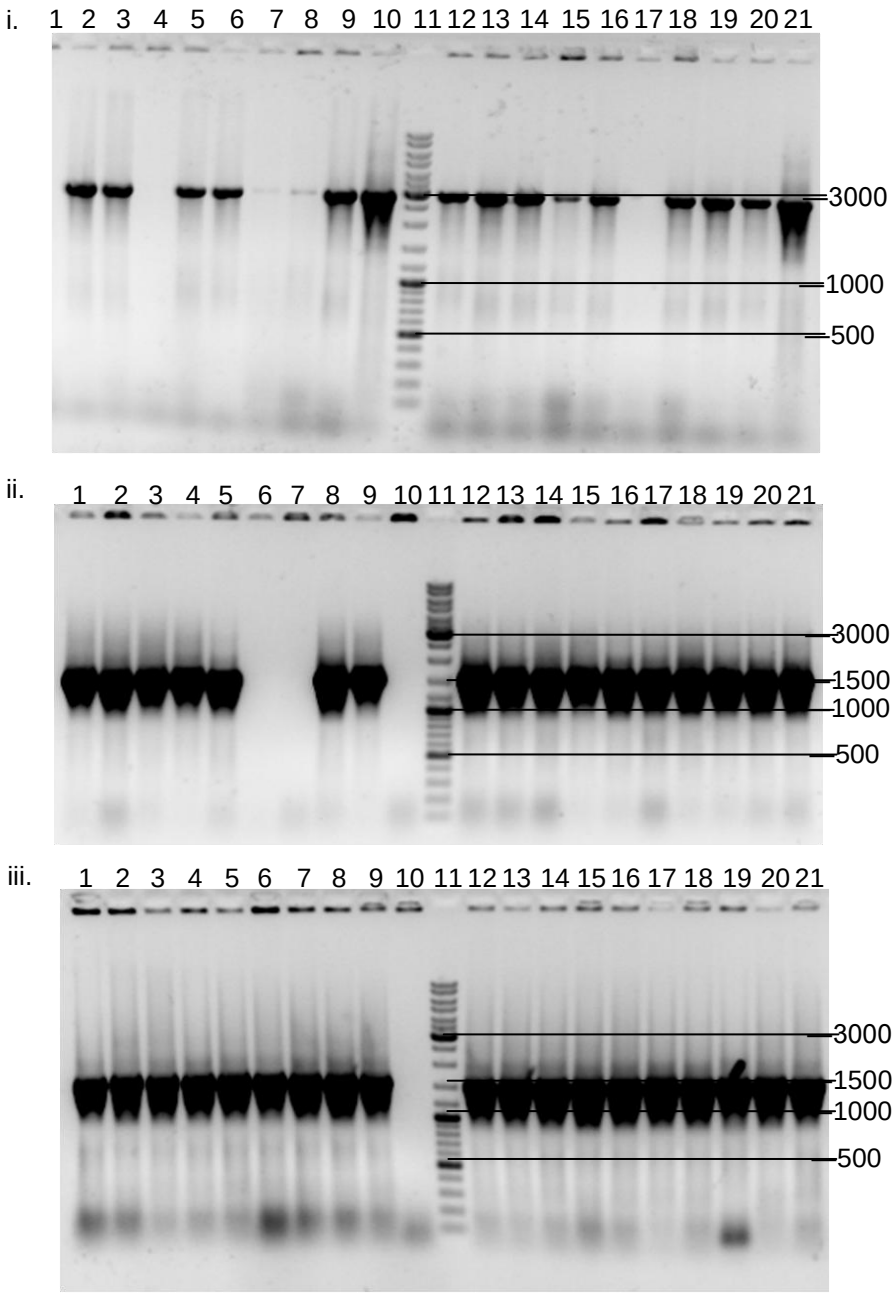

Figure A4: Day 4 of stability assessment of recombinant plasmid vaccines in *Salmonella enterica* serovar *typhimurium* SL3261 tested with a diagnostic colony PCR. i. Mucosal pCI-neo DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, ampicillin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, ampicillin. ii. VR1012 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin. iii. VR1020 mucosal DNA vaccine. Lane 1-10: 10 µl of colony PCR from selected colonies on TSA plates without the antibiotic, kanamycin. Lane 11: 5 µl DNA Ladder mix (Generuler^{TM}, Fermentas). Lane 12-21: 10 µl of colony PCR from selected colonies on TSA plates with the antibiotic, kanamycin.

Addendum B: Statistical analysis of ELISA results of the preliminary vaccine trial on a commercial ostrich farm in Oudtshoorn, Western Cape

Humoral immune response of ostriches against OppA

The immune response of the ostriches against the OppA protein was evaluated using ELISA as described in section 4.2.3.4 and the serum samples collected. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Serum - OppA
Oudsthoorn
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	359	57.063			
Treatment	3	5.919	1.973	14.500	0.0000
Time	2	2.416	1.208	8.880	0.0002
Treatment x Time	6	1.361	0.227	1.670	0.1284
Residual	348	47.367	0.136		

Grand mean = 0.728 R-squared = 0.1699 C.V. = 50.69%

LSD for Treatment = 0.1082 S.E.D = 0.055 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.13611

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
1	0.93	49.7	1	Salmonella Control
3	0.75	52.2	2	VR1020 Plasmid
4	0.63	51.6	3	Control
2	0.60	53.1	4	Salmonella + VR1020

LSD for Time = 0.0937 S.E.D = 0.0476 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.13611

Time
Averages

Level	--- Y ---	Cv	Rank	Week
1	0.80	51.3	1	0
2	0.77	53.4	2	3
3	0.61	56.9	3	6

LSD for Treatment*Time = 0.1874 S.E.D = 0.0953 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.13611

Two-way table for Treatment*Time, n=30

	1	2	3
1	0.961	0.990	0.829
2	0.750	0.590	0.453
3	0.814	0.891	0.554
4	0.687	0.596	0.621

A N A L Y S I S O F V A R I A N C E
Variable: WEIGHT

Source	df	SS	MS	F-value	Pr>F
Total	359	17242.88			
Treatment	3	1543.189	514.396	11.79	0.0000
Time	2	410.744	205.372	4.71	0.0096
Treatment x Time	6	101.732	16.955	0.39	0.8663
Residual	348	15187.21	43.641		

Grand mean = 41.192 R-squared = 0.1192 C.V. = 16.04%

LSD for Treatment = 1.9369 S.E.D = 0.9848 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 43.64141

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
1	43.55	13.8	1	Salmonella control
2	42.62	20.6	2	Salmonella + VR1020
3	40.39	13.6	3	VR1020 Plasmid
4	38.21	14.9	4	Control

LSD for Time = 1.6774 S.E.D = 0.8529 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 43.64141

Time
Averages

Level	--- Y ---	Cv	Rank	Time
3	42.7	24	1	6
2	40.52	11.7	2	3
1	40.35	9.3	3	0

LSD for Treatment*Time = 3.3548 S.E.D = 1.7057 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 43.64141

Two-way table for Treatment*Time, n=30

	1	2	3
1	42.38	42.277	45.98
2	41.337	41.91	44.627
3	39.8	40.207	41.163
4	37.9	37.963	39.027

The following table contains the input data, and is arranged in two columns with the ostrich identification number, Treatment received (Trt), Time in weeks, the response as measured by ELISA (Resp) and weights of the ostriches, read from the top to the bottom and left to right. The Treatments consist of *Salmonella enterica* serovar *typhimurium* SL3261 control (1), mucosal VR1020 recombinant vaccine (2), intramuscular injected VR1020 recombinant vaccine (3) and the control group that received no vaccinations (4). Following the data are the graphs drawn for each ostrich depicting the immune response measured over time.

Ostrich	Trt	Time	Resp	Weight
2700	1	0	1.759	46.6
2701	1	0	0.748	45.0
2702	1	0	0.883	43.0
2703	1	0	1.271	38.0
2704	1	0	1.243	49.0
2705	1	0	0.967	50.0
2706	1	0	0.765	39.0
2707	1	0	1.248	40.0
2708	1	0	1.282	48.0
2709	1	0	0.614	43.0
2710	1	0	0.722	38.0
2711	1	0	0.678	39.0
2712	1	0	0.157	39.0
2713	1	0	0.377	36.0
2714	1	0	1.144	34.0
2715	1	0	1.028	39.0
2716	1	0	0.592	42.0
2717	1	0	1.264	46.0
2718	1	0	0.228	39.0
2719	1	0	0.241	45.0
2720	1	0	1.230	51.0
2721	1	0	0.724	40.0
2722	1	0	1.387	39.0
2723	1	0	1.287	42.0
2724	1	0	2.465	43.0
2725	1	0	0.465	43.0
2726	1	0	0.723	39.0
2727	1	0	0.981	49.0
2728	1	0	1.124	47.0
2729	1	0	1.256	39.8
2730	2	0	1.360	44.1
2731	2	0	0.711	46.0
2732	2	0	0.675	41.0
2733	2	0	0.760	45.0
2734	2	0	0.851	47.0
2735	2	0	0.690	35.0
2736	2	0	0.300	40.0
2737	2	0	0.799	41.0
2738	2	0	0.354	34.0
2739	2	0	0.814	38.0
2740	2	0	0.495	48.0
2741	2	0	0.307	40.0
2742	2	0	0.820	42.0
2743	2	0	0.879	46.0
2744	2	0	1.032	36.0
2745	2	0	0.229	49.0
2746	2	0	0.333	41.0
2747	2	0	1.557	43.0
2748	2	0	0.573	41.0
2749	2	0	0.891	36.0
2750	2	0	1.176	38.0
2751	2	0	0.725	39.0
2752	2	0	0.470	40.0
2753	2	0	0.465	42.0
2754	2	0	1.305	44.0
2755	2	0	0.446	44.0
2756	2	0	0.436	43.0
2757	2	0	1.380	38.0
2758	2	0	0.596	36.0
2759	2	0	1.053	43.0
2760	3	0	0.991	40.0
2761	3	0	0.583	40.0
2762	3	0	0.594	38.0
2763	3	0	0.840	43.0
2764	3	0	0.410	41.0
2765	3	0	0.398	37.0
2766	3	0	0.858	44.0
2767	3	0	0.694	40.0
2768	3	0	0.697	39.0
2769	3	0	0.528	39.0
2770	3	0	1.117	37.0
2771	3	0	0.651	48.0
2772	3	0	1.230	37.0
2773	3	0	0.852	37.0
2774	3	0	1.535	43.0
2775	3	0	0.865	39.0
2776	3	0	1.180	37.0
2777	3	0	0.858	39.0
2778	3	0	1.667	41.0
2779	3	0	0.944	37.0
2780	3	0	0.454	40.0
2781	3	0	0.933	39.0
2782	3	0	0.287	39.0
2783	3	0	0.286	40.0
2784	3	0	0.755	41.0
2785	3	0	1.014	41.0
2786	3	0	0.363	41.0
2787	3	0	0.708	39.0
2788	3	0	0.227	39.0
2789	3	0	1.889	39.0
2790	4	0	0.527	38.0
2791	4	0	0.790	40.0
2792	4	0	0.887	36.0
2793	4	0	0.707	38.0
2794	4	0	0.903	39.0
2795	4	0	0.720	37.0
2796	4	0	0.649	40.0
2797	4	0	0.481	37.0
2798	4	0	0.208	36.0

2799	4	0	1.273	36.0
2800	4	0	1.002	37.0
2801	4	0	0.720	37.0
2802	4	0	0.887	36.0
2803	4	0	0.682	37.0
2804	4	0	0.372	35.0
2805	4	0	0.615	43.0
2806	4	0	1.645	38.0
2807	4	0	0.348	38.0
2808	4	0	0.639	45.0
2809	4	0	0.897	37.0
2810	4	0	1.308	38.0
2811	4	0	0.431	38.0
2812	4	0	0.570	40.0
2813	4	0	0.646	38.0
2814	4	0	1.292	37.0
2815	4	0	0.480	36.0
2816	4	0	0.111	42.0
2817	4	0	0.147	37.0
2818	4	0	0.492	36.0
2819	4	0	0.177	35.0
2700	1	3	1.938	50.5
2701	1	3	1.049	45.4
2702	1	3	1.186	43.0
2703	1	3	0.951	40.6
2704	1	3	1.736	55.5
2705	1	3	0.861	46.0
2706	1	3	1.566	39.2
2707	1	3	1.097	37.8
2708	1	3	0.511	42.0
2709	1	3	0.538	44.8
2710	1	3	0.404	40.2
2711	1	3	0.540	35.2
2712	1	3	0.482	37.2
2713	1	3	0.160	33.6
2714	1	3	1.150	34.4
2715	1	3	0.543	35.0
2716	1	3	0.640	35.6
2717	1	3	1.090	39.8
2718	1	3	0.322	43.8
2719	1	3	0.675	44.6
2720	1	3	0.849	56.5
2721	1	3	1.273	39.2
2722	1	3	1.279	43.6
2723	1	3	1.046	40.2
2724	1	3	1.097	42.8
2725	1	3	0.818	45.4
2726	1	3	1.158	40.8
2727	1	3	1.916	49.6
2728	1	3	1.602	44.4
2729	1	3	1.202	41.6

2730	2	3	1.106	43.2
2731	2	3	0.495	47.4
2732	2	3	0.540	42.8
2733	2	3	0.371	46.8
2734	2	3	0.656	52.0
2735	2	3	0.791	30.4
2736	2	3	0.437	38.8
2737	2	3	0.394	46.4
2738	2	3	0.371	36.4
2739	2	3	0.782	38.2
2740	2	3	0.390	49.2
2741	2	3	0.181	43.8
2742	2	3	0.739	42.4
2743	2	3	0.756	44.8
2744	2	3	0.974	34.8
2745	2	3	0.175	50.5
2746	2	3	0.172	41.2
2747	2	3	1.088	44.4
2748	2	3	0.746	36.8
2749	2	3	0.373	39.4
2750	2	3	0.531	40.2
2751	2	3	1.024	34.8
2752	2	3	0.857	39.0
2753	2	3	0.510	41.6
2754	2	3	0.262	40.6
2755	2	3	0.401	44.4
2756	2	3	0.357	45.2
2757	2	3	0.779	40.6
2758	2	3	0.703	40.2
2759	2	3	0.718	41.0
2760	3	3	1.140	39.4
2761	3	3	0.535	42.6
2762	3	3	0.747	37.0
2763	3	3	1.247	39.8
2764	3	3	1.039	41.2
2765	3	3	0.671	37.8
2766	3	3	0.855	40.6
2767	3	3	0.794	43.2
2768	3	3	0.740	43.2
2769	3	3	0.786	38.8
2770	3	3	1.074	43.8
2771	3	3	1.179	41.4
2772	3	3	1.970	40.8
2773	3	3	1.333	37.6
2774	3	3	0.833	46.6
2775	3	3	1.582	32.0
2776	3	3	1.352	37.8
2777	3	3	0.523	41.4
2778	3	3	0.745	44.6
2779	3	3	1.048	38.0
2780	3	3	0.430	41.8

2781	3	3	1.252	39.2
2782	3	3	0.348	37.6
2783	3	3	0.159	35.0
2784	3	3	0.247	40.8
2785	3	3	1.020	42.4
2786	3	3	0.405	41.0
2787	3	3	0.894	36.2
2788	3	3	0.713	44.4
2789	3	3	1.082	40.2
2790	4	3	0.537	37.8
2791	4	3	0.585	36.8
2792	4	3	0.369	32.4
2793	4	3	0.727	34.2
2794	4	3	0.505	39.0
2795	4	3	0.717	40.2
2796	4	3	0.000	36.2
2797	4	3	0.480	34.4
2798	4	3	0.205	41.4
2799	4	3	0.731	36.4
2800	4	3	1.149	37.0
2801	4	3	0.639	33.2
2802	4	3	0.844	36.4
2803	4	3	0.580	40.6
2804	4	3	0.308	36.6
2805	4	3	0.543	41.0
2806	4	3	1.257	35.4
2807	4	3	1.097	38.2
2808	4	3	0.613	44.4
2809	4	3	1.140	35.4
2810	4	3	0.635	38.6
2811	4	3	1.085	40.2
2812	4	3	0.736	43.4
2813	4	3	0.293	37.4
2814	4	3	0.291	34.0
2815	4	3	0.788	31.0
2816	4	3	0.053	47.0
2817	4	3	0.369	39.2
2818	4	3	0.332	40.0
2819	4	3	0.277	33.0
2700	1	6	1.745	51.5
2701	1	6	1.526	49.4
2702	1	6	1.462	44.0
2703	1	6	1.502	47.6
2704	1	6	1.200	63.0
2705	1	6	1.170	49.0
2706	1	6	1.387	42.4
2707	1	6	0.874	39.6
2708	1	6	1.022	43.6
2709	1	6	0.692	49.2
2710	1	6	0.980	41.8
2711	1	6	0.595	35.8
2712	1	6	0.434	39.4
2713	1	6	0.030	32.8
2714	1	6	0.811	40.4
2715	1	6	0.275	40.8
2716	1	6	0.452	37.8
2717	1	6	0.830	44.8
2718	1	6	0.319	48.2
2719	1	6	1.193	50.0
2720	1	6	0.453	64.0
2721	1	6	0.546	41.2
2722	1	6	0.542	45.8
2723	1	6	0.734	42.0
2724	1	6	0.385	48.0
2725	1	6	0.906	51.5
2726	1	6	0.294	47.0
2727	1	6	0.583	59.0
2728	1	6	1.060	46.6
2729	1	6	0.873	43.2
2730	2	6	0.870	41.8
2731	2	6	0.449	58.0
2732	2	6	.	.
2733	2	6	0.675	51.5
2734	2	6	0.210	62.5
2735	2	6	0.675	35.4
2736	2	6	0.201	45.0
2737	2	6	0.813	50.0
2738	2	6	0.283	35.0
2739	2	6	0.729	46.6
2740	2	6	0.293	61.0
2741	2	6	0.240	50.5
2742	2	6	0.603	49.8
2743	2	6	0.368	48.6
2744	2	6	0.488	32.0
2745	2	6	0.274	52.0
2746	2	6	0.321	53.5
2747	2	6	0.751	53.5
2748	2	6	.	.
2749	2	6	0.461	46.6
2750	2	6	0.158	46.0
2751	2	6	0.565	45.0
2752	2	6	0.491	47.6
2753	2	6	0.422	52.5
2754	2	6	0.728	47.8
2755	2	6	0.125	46.0
2756	2	6	0.386	47.4
2757	2	6	0.945	45.0
2758	2	6	0.346	47.4
2759	2	6	0.727	40.8
2760	3	6	0.889	42.0
2761	3	6	0.201	44.8
2762	3	6	0.051	39.8

2763	3	6	1.321	39.2
2764	3	6	0.352	49.0
2765	3	6	0.646	38.2
2766	3	6	0.414	45.4
2767	3	6	0.613	47.8
2768	3	6	0.824	46.8
2769	3	6	0.605	39.2
2770	3	6	0.654	42.0
2771	3	6	0.374	42.8
2772	3	6	0.779	40.4
2773	3	6	0.474	33.4
2774	3	6	0.255	45.2
2775	3	6	0.562	37.8
2776	3	6	0.704	40.3
2777	3	6	0.820	44.4
2778	3	6	0.305	46.0
2779	3	6	0.404	37.0
2780	3	6	0.693	44.2
2781	3	6	0.897	45.8
2782	3	6	.	.
2783	3	6	0.797	38.0
2784	3	6	0.306	43.4
2785	3	6	0.560	48.4
2786	3	6	0.394	42.6
2787	3	6	0.775	36.6
2788	3	6	0.195	46.6
2789	3	6	0.771	47.8
2790	4	6	0.242	42.4
2791	4	6	0.413	44.4
2792	4	6	0.436	35.0
2793	4	6	0.834	33.2
2794	4	6	.	.
2795	4	6	0.512	46.6
2796	4	6	0.654	36.0
2797	4	6	0.731	35.8
2798	4	6	0.440	48.6
2799	4	6	0.849	38.6
2800	4	6	0.747	46.2
2801	4	6	0.431	38.4
2802	4	6	0.783	35.6
2803	4	6	1.233	39.4
2804	4	6	0.557	37.2
2805	4	6	0.603	48.4
2806	4	6	0.775	40.2
2807	4	6	0.745	38.4
2808	4	6	1.084	48.0
2809	4	6	0.759	38.8
2810	4	6	0.476	43.8
2811	4	6	0.960	40.2
2812	4	6	0.973	43.0
2813	4	6	0.638	38.8
2814	4	6	1.041	43.0
2815	4	6	0.606	28.2
2816	4	6	0.213	47.6
2817	4	6	0.574	36.0
2818	4	6	0.135	42.6
2819	4	6	0.201	36.4

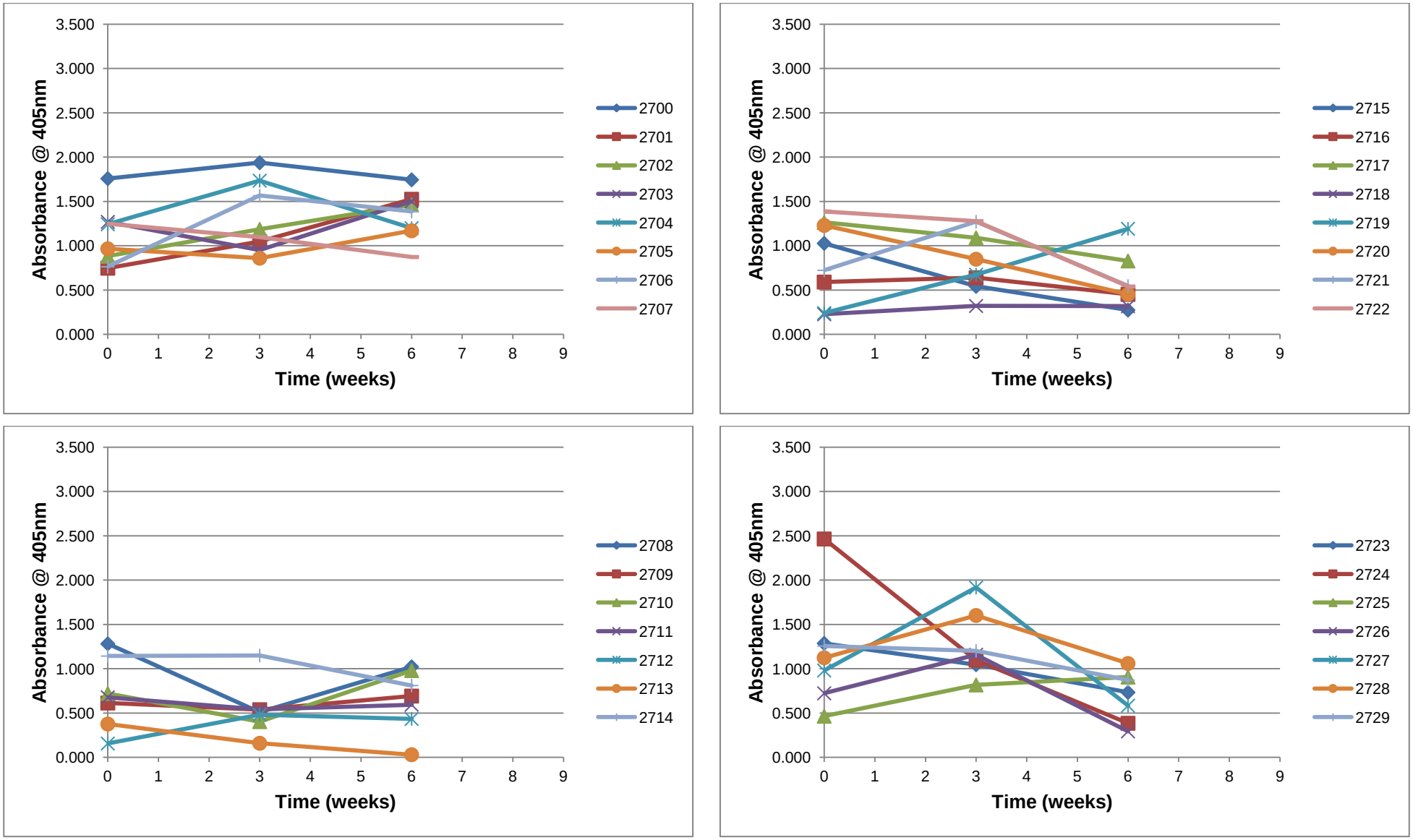

Figure B.1.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of *Salmonella enterica* serovar *typhimurium* SL3261 control

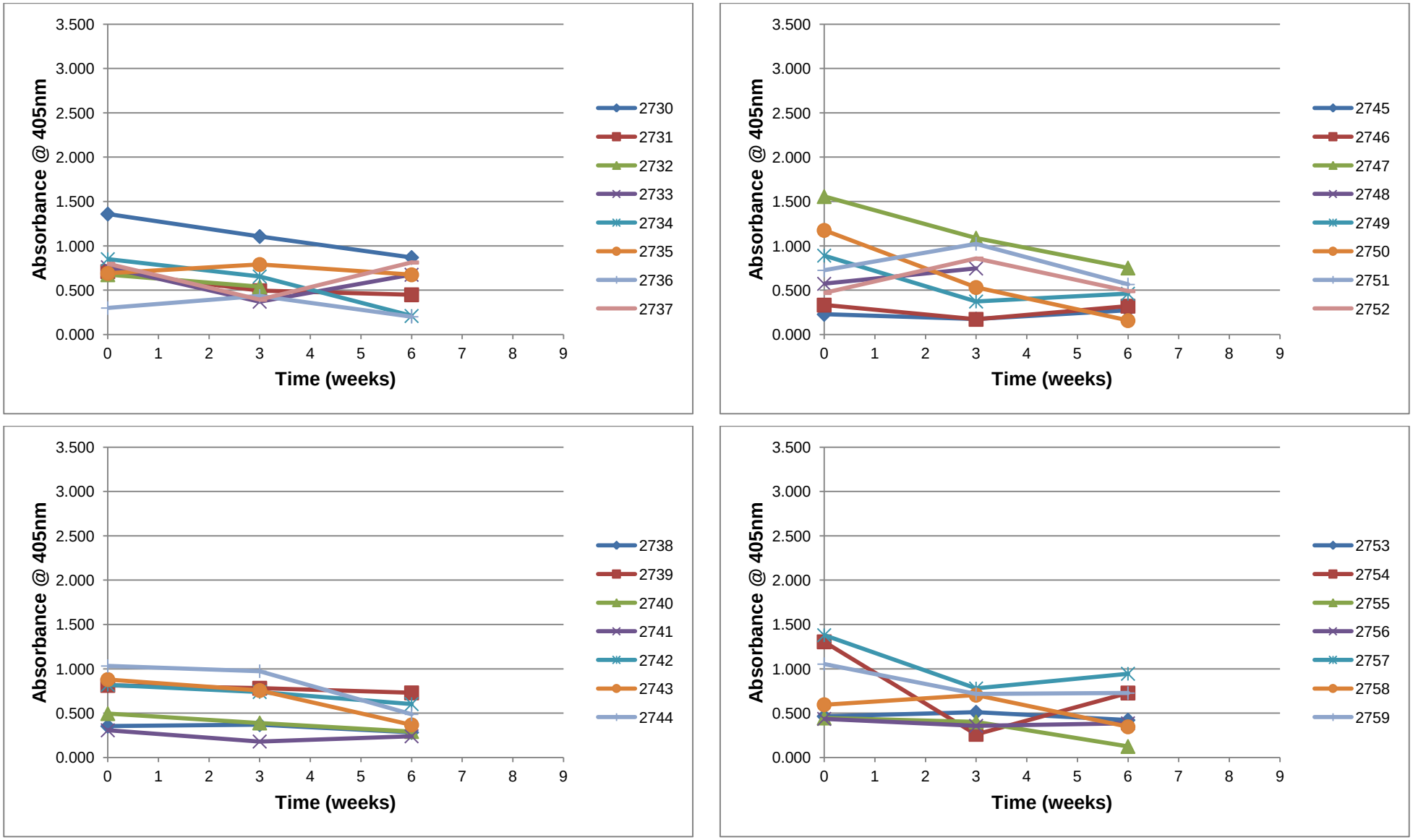

Figure B.2.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of the mucosal VR1020 recombinant vaccine

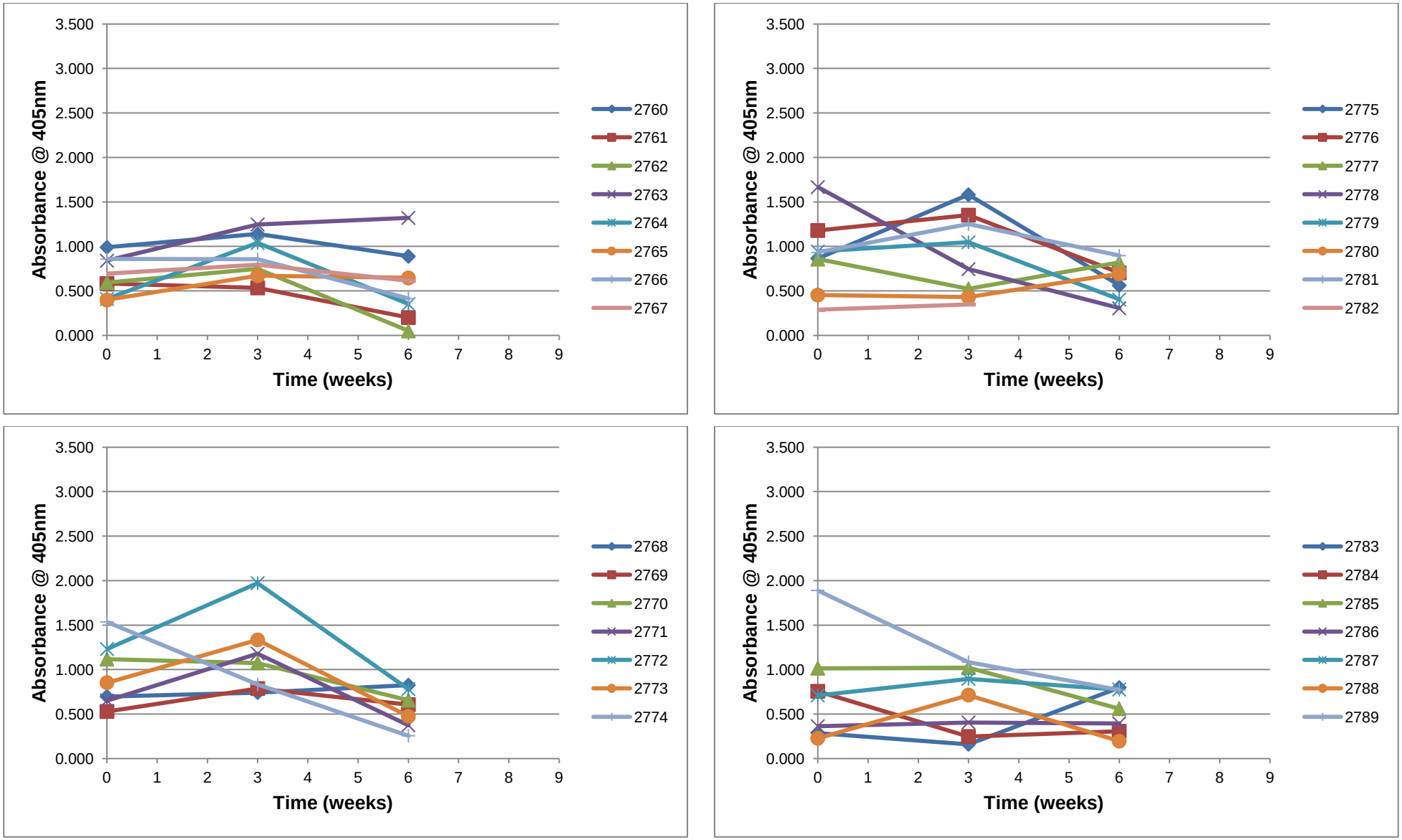

Figure B.3.: Immune responses elicited by ostriches that received 100 µg/ml of the intramuscular VR1020 recombinant vaccine

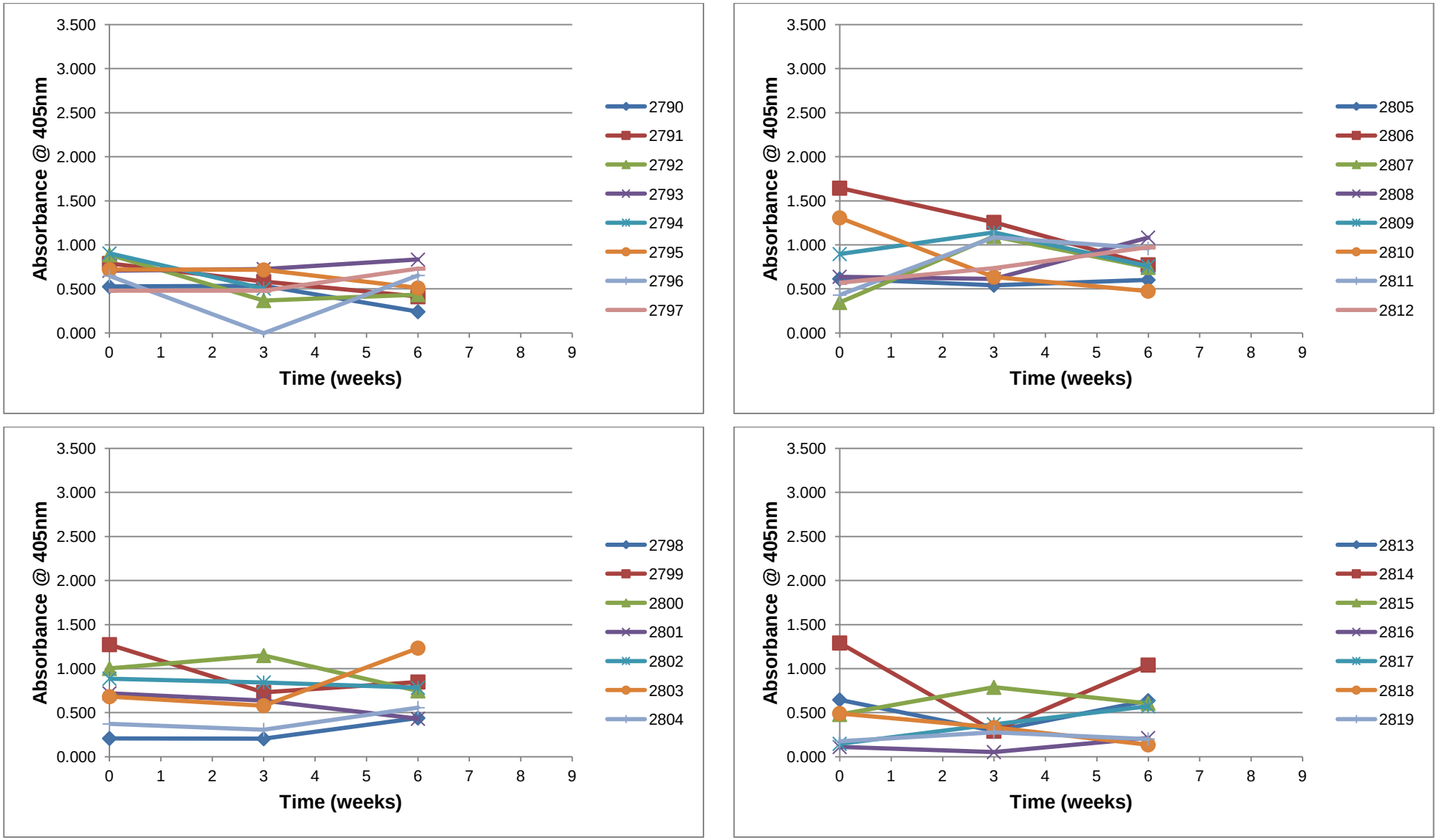

Figure B.4.: Immune responses elicited by ostriches in the control group that received no vaccinations

Mucosal immune response of ostriches against OppA

The mucosal immune response of the ostriches against the OppA protein was evaluated using ELISA as described in chapter 4.2.3.4 using the secondary antibody rabbit anti-ostrich IgA protein 1. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – OppA – IgA protein 1
Oudsthoorn
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	359	0.050			
Treatment (Treatment)	3	0.003	0.001	8.67	0.0000
Time (Time)	2	0.000	0.000	1.40	0.2474
Treatment x Time	6	0.001	0.000	1.19	0.3123
Residual	348	0.046	0.000		

Grand mean = 0.006 R-squared = 0.0936 C.V. = 203.9%

LSD for Treatment = 0.0034 S.E.D = 0.0017 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00013

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
1	0.01	174.5	1	Salmonella Control
3	0.01	184.7	2	VR1020 Plasmid
4	0.00	173.6	3	Control
2	0.00	303.4	4	Salmonella + VR1020

LSD for Time = 0.0029 S.E.D = 0.0015 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00013

Time
Averages

Level	--- Y ---	Cv	Rank	Week
1	0.01	233.6	1	0
2	0.01	169.0	2	3
3	0.00	206.1	3	6

LSD for Treatment*Time = 0.0058 S.E.D = 0.0030 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00013

Two-way table for Treatment*Time, n=30

	1	2	3
1	0.015	0.008	0.008
2	0.002	0.002	0.001
3	0.005	0.007	0.006
4	0.005	0.006	0.002

In the following table, the data is arranged in two columns with the ostrich identification number, Treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The Treatments consist of *Salmonella enterica* serovar *typhimurium* SL3261 control (1), mucosal VR1020 recombinant vaccine (2), intramuscular injected VR1020 recombinant vaccine (3) and the control group that received no vaccinations (4). Following the data are the graphs drawn for each ostrich depicting the immune response measured over time.

Ostrich	Trt	Time	Resp	Weight
2700	1	0	0.038	46.6
2701	1	0	0.066	45.0
2702	1	0	0.031	43.0
2703	1	0	0.038	38.0
2704	1	0	0.006	49.0
2705	1	0	0.004	50.0
2706	1	0	0.001	39.0
2707	1	0	0.008	40.0
2708	1	0	0.011	48.0
2709	1	0	0.012	43.0
2710	1	0	0.018	38.0
2711	1	0	0.130	39.0
2712	1	0	0.017	39.0
2713	1	0	0.010	36.0
2714	1	0	0.000	34.0
2715	1	0	0.002	39.0
2716	1	0	0.000	42.0
2717	1	0	0.000	46.0
2718	1	0	0.000	39.0
2719	1	0	0.000	45.0
2720	1	0	0.000	51.0
2721	1	0	0.011	40.0
2722	1	0	0.002	39.0
2723	1	0	0.000	42.0
2724	1	0	0.024	43.0
2725	1	0	0.000	43.0
2726	1	0	0.000	39.0
2727	1	0	0.000	49.0
2728	1	0	0.002	47.0
2729	1	0	0.006	39.8
2730	2	0	0.002	44.1
2731	2	0	0.003	46.0
2732	2	0	0.011	41.0
2733	2	0	0.002	45.0
2734	2	0	0.003	47.0
2735	2	0	0.000	35.0
2736	2	0	0.000	40.0
2737	2	0	0.000	41.0
2738	2	0	0.000	34.0
2739	2	0	0.000	38.0
2740	2	0	0.000	48.0
2741	2	0	0.000	40.0
2742	2	0	0.000	42.0
2743	2	0	0.000	46.0
2744	2	0	0.000	36.0
2745	2	0	0.004	49.0
2746	2	0	0.000	41.0
2747	2	0	0.000	43.0
2748	2	0	0.023	41.0
2749	2	0	0.000	36.0
2750	2	0	0.000	38.0
2751	2	0	0.000	39.0
2752	2	0	0.000	40.0
2753	2	0	0.000	42.0
2754	2	0	0.000	44.0
2755	2	0	0.000	44.0
2756	2	0	0.020	43.0
2757	2	0	0.000	38.0
2758	2	0	0.000	36.0
2759	2	0	0.000	43.0
2760	3	0	0.008	40.0
2761	3	0	0.006	40.0
2762	3	0	0.000	38.0
2763	3	0	0.000	43.0
2764	3	0	0.023	41.0
2765	3	0	0.000	37.0
2766	3	0	0.000	44.0
2767	3	0	0.001	40.0
2768	3	0	0.000	39.0
2769	3	0	0.000	39.0
2770	3	0	0.000	37.0
2771	3	0	0.025	48.0
2772	3	0	0.000	37.0
2773	3	0	0.000	37.0
2774	3	0	0.000	43.0
2775	3	0	0.000	39.0
2776	3	0	0.000	37.0
2777	3	0	0.000	39.0
2778	3	0	0.000	41.0

2779	3	0	0.000	37.0
2780	3	0	0.000	40.0
2781	3	0	0.000	39.0
2782	3	0	0.023	39.0
2783	3	0	0.008	40.0
2784	3	0	0.002	41.0
2785	3	0	0.037	41.0
2786	3	0	0.003	41.0
2787	3	0	0.012	39.0
2788	3	0	0.000	39.0
2789	3	0	0.006	39.0
2790	4	0	0.015	38.0
2791	4	0	0.000	40.0
2792	4	0	0.008	36.0
2793	4	0	0.003	38.0
2794	4	0	0.008	39.0
2795	4	0	0.012	37.0
2796	4	0	0.001	40.0
2797	4	0	0.015	37.0
2798	4	0	0.013	36.0
2799	4	0	0.001	36.0
2800	4	0	0.000	37.0
2801	4	0	0.011	37.0
2802	4	0	0.012	36.0
2803	4	0	0.000	37.0
2804	4	0	0.000	35.0
2805	4	0	0.013	43.0
2806	4	0	0.000	38.0
2807	4	0	0.000	38.0
2808	4	0	0.019	45.0
2809	4	0	0.000	37.0
2810	4	0	0.000	38.0
2811	4	0	0.004	38.0
2812	4	0	0.006	40.0
2813	4	0	0.000	38.0
2814	4	0	0.001	37.0
2815	4	0	0.000	36.0
2816	4	0	0.025	42.0
2817	4	0	0.012	37.0
2818	4	0	0.000	36.0
2819	4	0	0.000	35.0
2700	1	3	0.011	50.5
2701	1	3	0.012	45.4
2702	1	3	0.004	43.0
2703	1	3	0.009	40.6
2704	1	3	0.041	55.5
2705	1	3	0.019	46.0
2706	1	3	0.019	39.2
2707	1	3	0.007	37.8
2708	1	3	0.009	42.0
2709	1	3	0.012	44.8

2710	1	3	0.013	40.2
2711	1	3	0.016	35.2
2712	1	3	0.014	37.2
2713	1	3	0.014	33.6
2714	1	3	0.000	34.4
2715	1	3	0.006	35.0
2716	1	3	0.004	35.6
2717	1	3	0.000	39.8
2718	1	3	0.000	43.8
2719	1	3	0.000	44.6
2720	1	3	0.000	56.5
2721	1	3	0.000	39.2
2722	1	3	0.000	43.6
2723	1	3	0.000	40.2
2724	1	3	0.019	42.8
2725	1	3	0.000	45.4
2726	1	3	0.000	40.8
2727	1	3	0.000	49.6
2728	1	3	0.004	44.4
2729	1	3	0.017	41.6
2730	2	3	0.005	43.2
2731	2	3	0.005	47.4
2732	2	3	0.010	42.8
2733	2	3	0.000	46.8
2734	2	3	0.000	52.0
2735	2	3	0.010	30.4
2736	2	3	0.000	38.8
2737	2	3	0.000	46.4
2738	2	3	0.000	36.4
2739	2	3	0.000	38.2
2740	2	3	0.000	49.2
2741	2	3	0.000	43.8
2742	2	3	0.000	42.4
2743	2	3	0.000	44.8
2744	2	3	0.010	34.8
2745	2	3	0.004	50.5
2746	2	3	0.016	41.2
2747	2	3	0.000	44.4
2748	2	3	0.005	36.8
2749	2	3	0.001	39.4
2750	2	3	0.000	40.2
2751	2	3	0.000	34.8
2752	2	3	0.000	39.0
2753	2	3	0.000	41.6
2754	2	3	0.000	40.6
2755	2	3	0.000	44.4
2756	2	3	0.004	45.2
2757	2	3	0.000	40.6
2758	2	3	0.000	40.2
2759	2	3	0.000	41.0
2760	3	3	0.009	39.4

2761	3	3	0.006	42.6
2762	3	3	0.000	37.0
2763	3	3	0.000	39.8
2764	3	3	0.020	41.2
2765	3	3	0.000	37.8
2766	3	3	0.002	40.6
2767	3	3	0.005	43.2
2768	3	3	0.000	43.2
2769	3	3	0.015	38.8
2770	3	3	0.000	43.8
2771	3	3	0.055	41.4
2772	3	3	0.000	40.8
2773	3	3	0.000	37.6
2774	3	3	0.000	46.6
2775	3	3	0.000	32.0
2776	3	3	0.000	37.8
2777	3	3	0.000	41.4
2778	3	3	0.001	44.6
2779	3	3	0.039	38.0
2780	3	3	0.008	41.8
2781	3	3	0.000	39.2
2782	3	3	0.012	37.6
2783	3	3	0.000	35.0
2784	3	3	0.007	40.8
2785	3	3	0.006	42.4
2786	3	3	0.018	41.0
2787	3	3	0.003	36.2
2788	3	3	0.000	44.4
2789	3	3	0.000	40.2
2790	4	3	0.002	37.8
2791	4	3	0.000	36.8
2792	4	3	0.002	32.4
2793	4	3	0.004	34.2
2794	4	3	0.000	39.0
2795	4	3	0.012	40.2
2796	4	3	0.006	36.2
2797	4	3	0.008	34.4
2798	4	3	0.011	41.4
2799	4	3	0.000	36.4
2800	4	3	0.002	37.0
2801	4	3	0.007	33.2
2802	4	3	0.005	36.4
2803	4	3	0.000	40.6
2804	4	3	0.013	36.6
2805	4	3	0.011	41.0
2806	4	3	0.000	35.4
2807	4	3	0.006	38.2
2808	4	3	0.005	44.4
2809	4	3	0.000	35.4
2810	4	3	0.007	38.6
2811	4	3	0.008	40.2
2812	4	3	0.004	43.4
2813	4	3	0.000	37.4
2814	4	3	0.002	34.0
2815	4	3	0.012	31.0
2816	4	3	0.011	47.0
2817	4	3	0.055	39.2
2818	4	3	0.000	40.0
2819	4	3	0.000	33.0
2700	1	6	0.014	51.5
2701	1	6	0.011	49.4
2702	1	6	0.015	44.0
2703	1	6	0.005	47.6
2704	1	6	0.014	63.0
2705	1	6	0.016	49.0
2706	1	6	0.027	42.4
2707	1	6	0.006	39.6
2708	1	6	0.006	43.6
2709	1	6	0.007	49.2
2710	1	6	0.015	41.8
2711	1	6	0.030	35.8
2712	1	6	0.029	39.4
2713	1	6	0.014	32.8
2714	1	6	0.000	40.4
2715	1	6	0.000	40.8
2716	1	6	0.000	37.8
2717	1	6	0.000	44.8
2718	1	6	0.000	48.2
2719	1	6	0.000	50.0
2720	1	6	0.000	64.0
2721	1	6	0.000	41.2
2722	1	6	0.000	45.8
2723	1	6	0.006	42.0
2724	1	6	0.001	48.0
2725	1	6	0.000	51.5
2726	1	6	0.000	47.0
2727	1	6	0.000	59.0
2728	1	6	0.000	46.6
2729	1	6	0.006	43.2
2730	2	6	0.000	41.8
2731	2	6	0.000	58.0
2732	2	6	.	.
2733	2	6	0.000	51.5
2734	2	6	0.000	62.5
2735	2	6	0.020	35.4
2736	2	6	0.000	45.0
2737	2	6	0.000	50.0
2738	2	6	0.000	35.0
2739	2	6	0.000	46.6
2740	2	6	0.000	61.0
2741	2	6	0.000	50.5
2742	2	6	0.001	49.8

2743	2	6	0.000	48.6
2744	2	6	0.000	32.0
2745	2	6	0.000	52.0
2746	2	6	0.000	53.5
2747	2	6	0.000	53.5
2748	2	6	.	.
2749	2	6	0.001	46.6
2750	2	6	0.000	46.0
2751	2	6	0.000	45.0
2752	2	6	0.000	47.6
2753	2	6	0.000	52.5
2754	2	6	0.000	47.8
2755	2	6	0.000	46.0
2756	2	6	0.004	47.4
2757	2	6	0.004	45.0
2758	2	6	0.000	47.4
2759	2	6	0.000	40.8
2760	3	6	0.000	42.0
2761	3	6	0.002	44.8
2762	3	6	0.012	39.8
2763	3	6	0.056	39.2
2764	3	6	0.024	49.0
2765	3	6	0.000	38.2
2766	3	6	0.011	45.4
2767	3	6	0.005	47.8
2768	3	6	0.000	46.8
2769	3	6	0.005	39.2
2770	3	6	0.011	42.0
2771	3	6	0.002	42.8
2772	3	6	0.000	40.4
2773	3	6	0.000	33.4
2774	3	6	0.000	45.2
2775	3	6	0.000	37.8
2776	3	6	0.000	40.3
2777	3	6	0.000	44.4
2778	3	6	0.000	46.0
2779	3	6	0.031	37.0
2780	3	6	0.015	44.2
2781	3	6	0.000	45.8
2782	3	6	.	.
2783	3	6	0.000	38.0
2784	3	6	0.009	43.4
2785	3	6	0.010	48.4
2786	3	6	0.000	42.6
2787	3	6	0.009	36.6
2788	3	6	0.000	46.6
2789	3	6	0.000	47.8
2790	4	6	0.000	42.4
2791	4	6	0.001	44.4
2792	4	6	0.001	35.0
2793	4	6	0.007	33.2
2794	4	6	.	.
2795	4	6	0.004	46.6
2796	4	6	0.000	36.0
2797	4	6	0.000	35.8
2798	4	6	0.007	48.6
2799	4	6	0.000	38.6
2800	4	6	0.006	46.2
2801	4	6	0.000	38.4
2802	4	6	0.000	35.6
2803	4	6	0.000	39.4
2804	4	6	0.000	37.2
2805	4	6	0.000	48.4
2806	4	6	0.000	40.2
2807	4	6	0.000	38.4
2808	4	6	0.000	48.0
2809	4	6	0.000	38.8
2810	4	6	0.003	43.8
2811	4	6	0.007	40.2
2812	4	6	0.007	43.0
2813	4	6	0.008	38.8
2814	4	6	0.007	43.0
2815	4	6	0.000	28.2
2816	4	6	0.001	47.6
2817	4	6	0.000	36.0
2818	4	6	0.003	42.6
2819	4	6	0.000	36.4

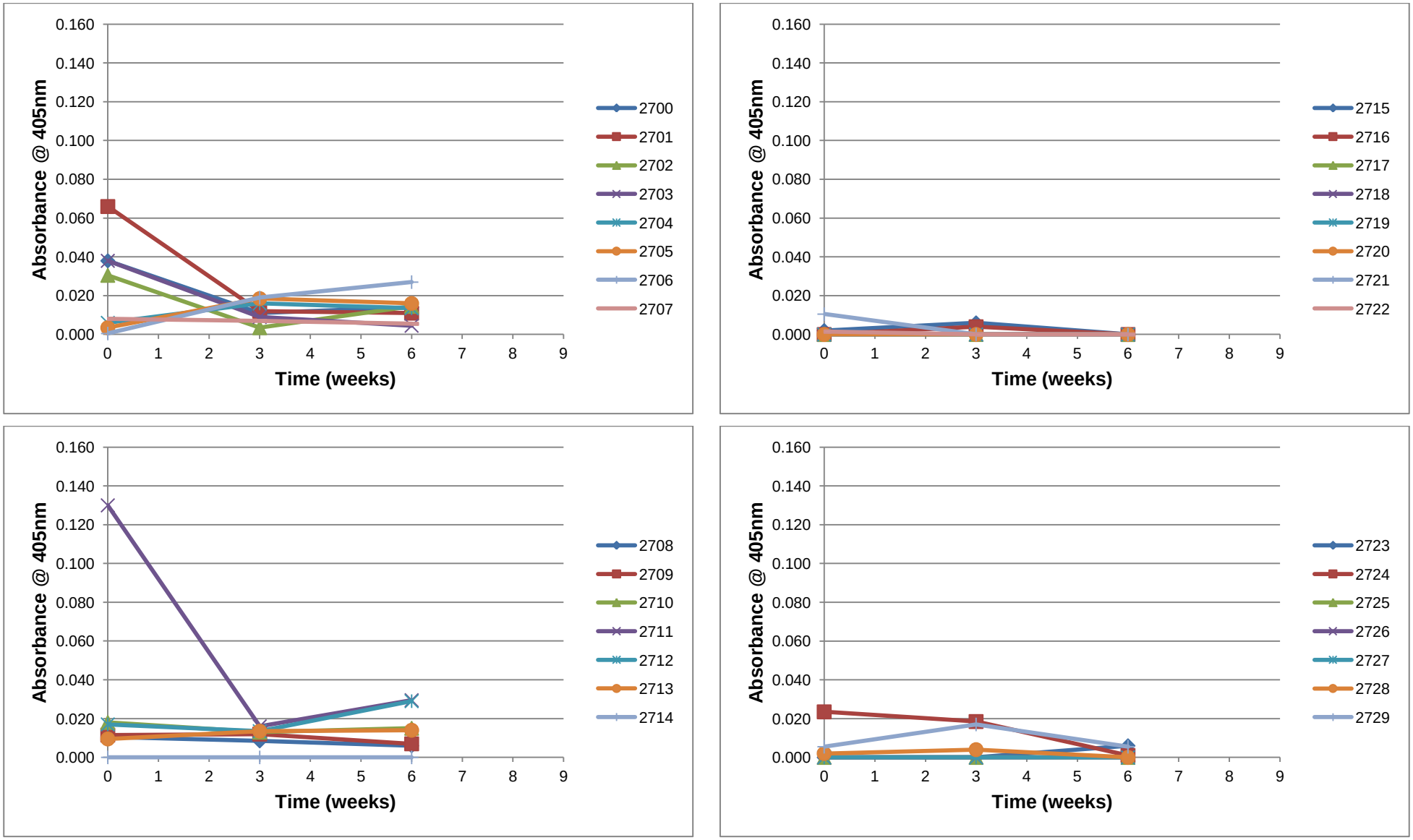

Figure B.5.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of *Salmonella enterica* serovar *typhimurium* SL3261 control

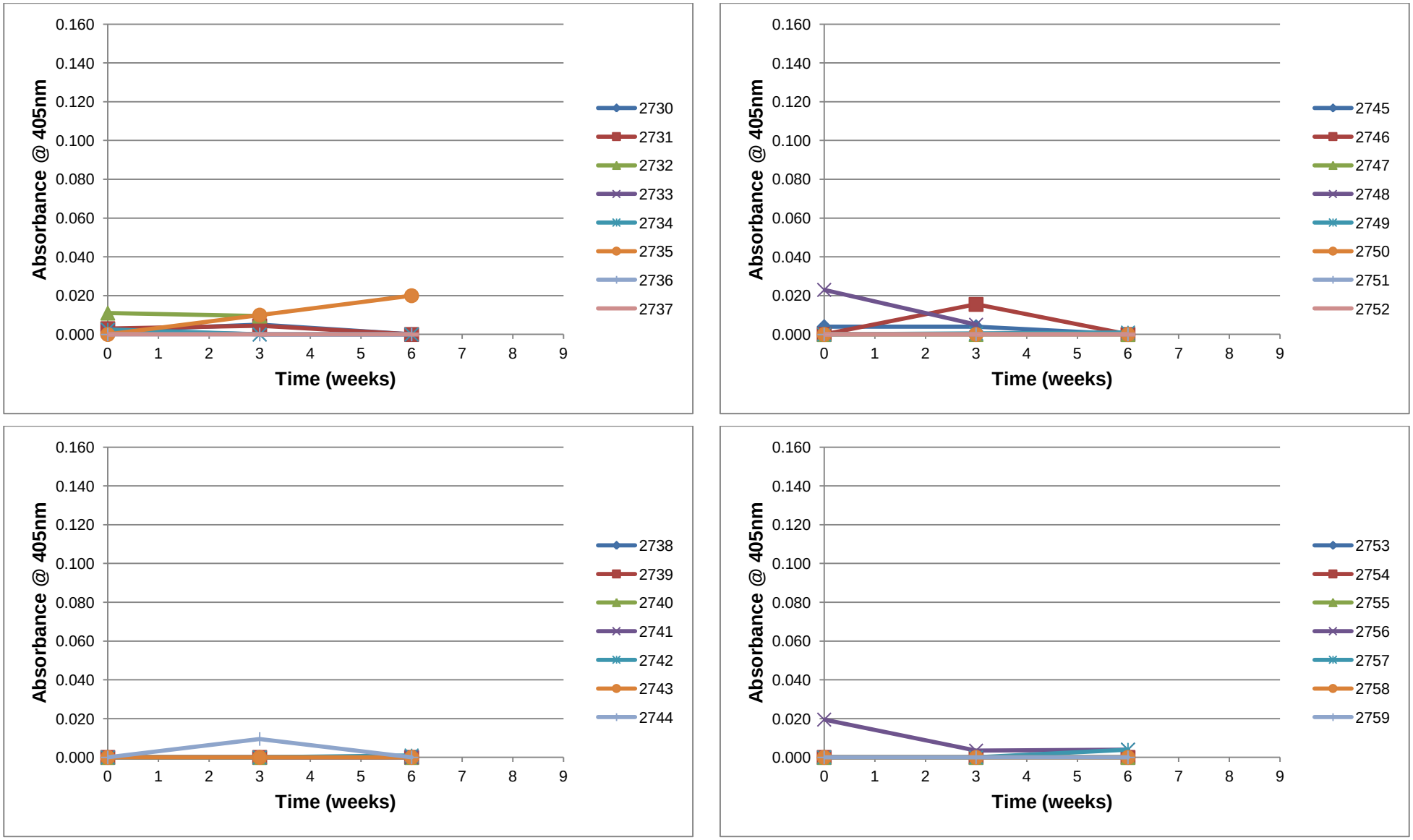

Figure B.6.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of the mucosal VR1020 recombinant vaccine

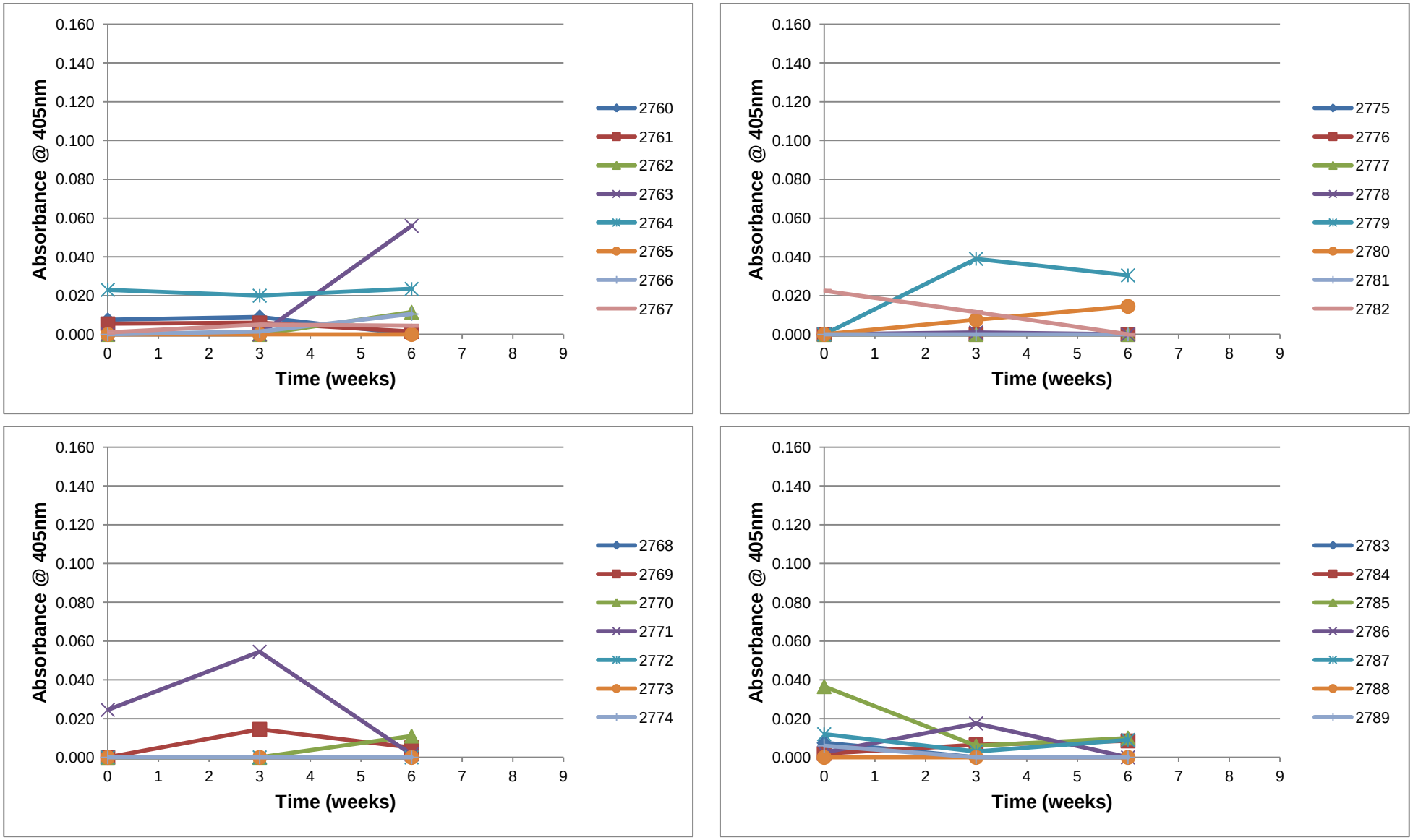

Figure B.7.: Immune responses elicited by ostriches that received 100 µg/ml of the intramuscular VR1020 recombinant vaccine

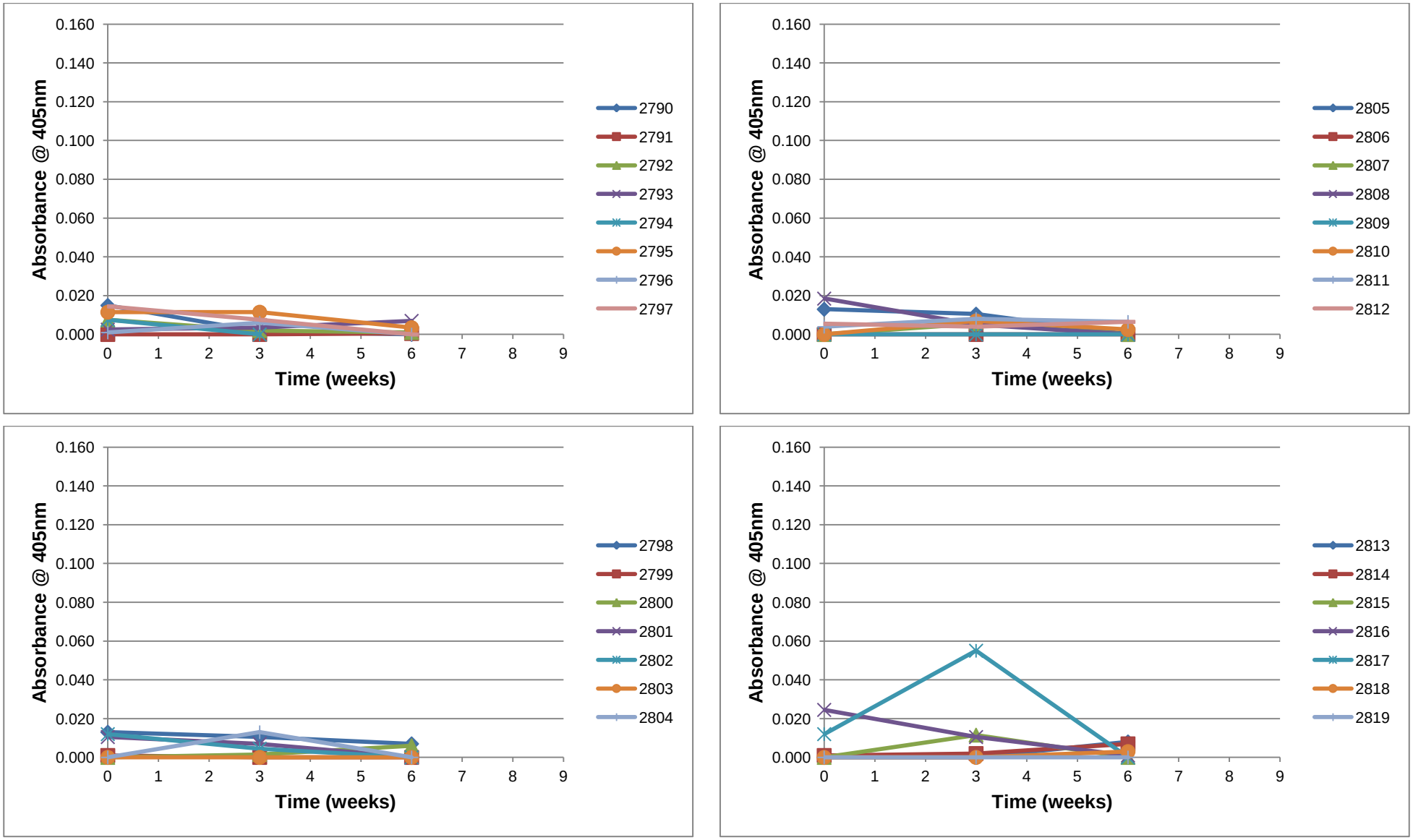

Figure B.8.: Immune responses elicited by ostriches in the control group that received no vaccinations

Mucosal immune response of ostriches against OppA

The mucosal immune response of the ostriches against the OppA protein was evaluated using ELISA as described in chapter 4.2.3.4 using the swab samples collected and rabbit anti-ostrich IgA protein 2 as the biotinylated secondary antibody. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – OppA – IgA protein 2
Oudsthoorn
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	359	0.063			
Treatment (Treatment)	3	0.001	0.000	1.37	0.2527
Time (Time)	2	0.000	0.000	0.63	0.5329
Treatment x Time	6	0.001	0.000	0.73	0.6294
Residual	348	0.061	0.000		

Grand mean = 0.007 R-squared = 0.0272 C.V. = 191.7%

LSD for Treatment = 0.0039 S.E.D = 0.0020 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00018

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
1	0.01	191.1	1	Salmonella Control
4	0.01	183.5	2	Control
3	0.01	157.9	3	VR1020 Plasmid
2	0.00	230.0	4	Salmonella + VR1020

LSD for Time = 0.0034 S.E.D = 0.0017 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00018

Time
Averages

Level	--- Y ---	Cv	Rank	Week
1	0.01	205.5	1	0
2	0.01	152.3	2	3
3	0.01	209.6	3	6

LSD for Treatment*Time = 0.0067 S.E.D = 0.0034 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00018

Two-way table for Treatment*Time, n=30

	1	2	3
1	0.012	0.008	0.006
2	0.006	0.006	0.002
3	0.006	0.008	0.007
4	0.007	0.006	0.008

The following table the data is arranged in two columns with the ostrich identification number, Treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The Treatments consist of *Salmonella enterica* serovar *typhimurium* SL3261 control (1), mucosal VR1020 recombinant vaccine (2), intramuscular injected VR1020 recombinant vaccine (3) and the control group that received no vaccinations (4). Following the data are the graphs drawn for each ostrich depicting the immune response measured over time.

Ostrich	Trt	Time	Resp	Weight
2700	1	0	0.011	46.6
2701	1	0	0.021	45.0
2702	1	0	0.030	43.0
2703	1	0	0.002	38.0
2704	1	0	0.000	49.0
2705	1	0	0.000	50.0
2706	1	0	0.013	39.0
2707	1	0	0.000	40.0
2708	1	0	0.000	48.0
2709	1	0	0.024	43.0
2710	1	0	0.015	38.0
2711	1	0	0.022	39.0
2712	1	0	0.106	39.0
2713	1	0	0.029	36.0
2714	1	0	0.002	34.0
2715	1	0	0.008	39.0
2716	1	0	0.000	42.0
2717	1	0	0.000	46.0
2718	1	0	0.000	39.0
2719	1	0	0.001	45.0
2720	1	0	0.010	51.0
2721	1	0	0.010	40.0
2722	1	0	0.069	39.0
2723	1	0	0.000	42.0
2724	1	0	0.000	43.0
2725	1	0	0.000	43.0
2726	1	0	0.000	39.0
2727	1	0	0.000	49.0
2728	1	0	0.000	47.0
2729	1	0	0.000	39.8
2730	2	0	0.012	44.1
2731	2	0	0.003	46.0
2732	2	0	0.009	41.0
2733	2	0	0.077	45.0
2734	2	0	0.003	47.0
2735	2	0	0.002	35.0
2736	2	0	0.000	40.0
2737	2	0	0.000	41.0
2738	2	0	0.000	34.0
2739	2	0	0.000	38.0
2740	2	0	0.000	48.0
2741	2	0	0.000	40.0
2742	2	0	0.000	42.0
2743	2	0	0.019	46.0
2744	2	0	0.000	36.0
2745	2	0	0.000	49.0
2746	2	0	0.000	41.0
2747	2	0	0.008	43.0
2748	2	0	0.019	41.0
2749	2	0	0.000	36.0
2750	2	0	0.000	38.0
2751	2	0	0.000	39.0
2752	2	0	0.000	40.0
2753	2	0	0.012	42.0
2754	2	0	0.000	44.0
2755	2	0	0.000	44.0
2756	2	0	0.000	43.0
2757	2	0	0.000	38.0
2758	2	0	0.016	36.0
2759	2	0	0.000	43.0
2760	3	0	0.017	40.0
2761	3	0	0.000	40.0
2762	3	0	0.013	38.0
2763	3	0	0.019	43.0
2764	3	0	0.001	41.0
2765	3	0	0.000	37.0
2766	3	0	0.000	44.0
2767	3	0	0.005	40.0
2768	3	0	0.000	39.0
2769	3	0	0.000	39.0
2770	3	0	0.000	37.0
2771	3	0	0.002	48.0
2772	3	0	0.000	37.0
2773	3	0	0.000	37.0
2774	3	0	0.004	43.0
2775	3	0	0.000	39.0
2776	3	0	0.000	37.0

2777	3	0	0.000	39.0
2778	3	0	0.000	41.0
2779	3	0	0.008	37.0
2780	3	0	0.000	40.0
2781	3	0	0.000	39.0
2782	3	0	0.028	39.0
2783	3	0	0.000	40.0
2784	3	0	0.000	41.0
2785	3	0	0.037	41.0
2786	3	0	0.036	41.0
2787	3	0	0.002	39.0
2788	3	0	0.003	39.0
2789	3	0	0.009	39.0
2790	4	0	0.001	38.0
2791	4	0	0.000	40.0
2792	4	0	0.000	36.0
2793	4	0	0.000	38.0
2794	4	0	0.021	39.0
2795	4	0	0.013	37.0
2796	4	0	0.006	40.0
2797	4	0	0.000	37.0
2798	4	0	0.002	36.0
2799	4	0	0.000	36.0
2800	4	0	0.000	37.0
2801	4	0	0.000	37.0
2802	4	0	0.006	36.0
2803	4	0	0.000	37.0
2804	4	0	0.000	35.0
2805	4	0	0.000	43.0
2806	4	0	0.005	38.0
2807	4	0	0.021	38.0
2808	4	0	0.008	45.0
2809	4	0	0.002	37.0
2810	4	0	0.027	38.0
2811	4	0	0.000	38.0
2812	4	0	0.000	40.0
2813	4	0	0.011	38.0
2814	4	0	0.000	37.0
2815	4	0	0.000	36.0
2816	4	0	0.000	42.0
2817	4	0	0.025	37.0
2818	4	0	0.019	36.0
2819	4	0	0.017	35.0
2700	1	3	0.009	50.5
2701	1	3	0.031	45.4
2702	1	3	0.041	43.0
2703	1	3	0.006	40.6
2704	1	3	0.011	55.5
2705	1	3	0.042	46.0
2706	1	3	0.015	39.2
2707	1	3	0.000	37.8
2708	1	3	0.013	42.0
2709	1	3	0.005	44.8
2710	1	3	0.017	40.2
2711	1	3	0.000	35.2
2712	1	3	0.011	37.2
2713	1	3	0.008	33.6
2714	1	3	0.000	34.4
2715	1	3	0.004	35.0
2716	1	3	0.007	35.6
2717	1	3	0.000	39.8
2718	1	3	0.000	43.8
2719	1	3	0.001	44.6
2720	1	3	0.005	56.5
2721	1	3	0.000	39.2
2722	1	3	0.000	43.6
2723	1	3	0.000	40.2
2724	1	3	0.004	42.8
2725	1	3	0.000	45.4
2726	1	3	0.000	40.8
2727	1	3	0.000	49.6
2728	1	3	0.000	44.4
2729	1	3	0.002	41.6
2730	2	3	0.006	43.2
2731	2	3	0.000	47.4
2732	2	3	0.001	42.8
2733	2	3	0.040	46.8
2734	2	3	0.000	52.0
2735	2	3	0.000	30.4
2736	2	3	0.000	38.8
2737	2	3	0.000	46.4
2738	2	3	0.000	36.4
2739	2	3	0.000	38.2
2740	2	3	0.000	49.2
2741	2	3	0.000	43.8
2742	2	3	0.000	42.4
2743	2	3	0.031	44.8
2744	2	3	0.000	34.8
2745	2	3	0.011	50.5
2746	2	3	0.000	41.2
2747	2	3	0.000	44.4
2748	2	3	0.024	36.8
2749	2	3	0.000	39.4
2750	2	3	0.000	40.2
2751	2	3	0.000	34.8
2752	2	3	0.015	39.0
2753	2	3	0.025	41.6
2754	2	3	0.000	40.6
2755	2	3	0.000	44.4
2756	2	3	0.012	45.2
2757	2	3	0.007	40.6
2758	2	3	0.014	40.2

2759	2	3	0.014	41.0
2760	3	3	0.000	39.4
2761	3	3	0.009	42.6
2762	3	3	0.014	37.0
2763	3	3	0.000	39.8
2764	3	3	0.003	41.2
2765	3	3	0.000	37.8
2766	3	3	0.005	40.6
2767	3	3	0.003	43.2
2768	3	3	0.012	43.2
2769	3	3	0.025	38.8
2770	3	3	0.000	43.8
2771	3	3	0.020	41.4
2772	3	3	0.000	40.8
2773	3	3	0.000	37.6
2774	3	3	0.000	46.6
2775	3	3	0.000	32.0
2776	3	3	0.000	37.8
2777	3	3	0.000	41.4
2778	3	3	0.000	44.6
2779	3	3	0.008	38.0
2780	3	3	0.025	41.8
2781	3	3	0.014	39.2
2782	3	3	0.024	37.6
2783	3	3	0.005	35.0
2784	3	3	0.040	40.8
2785	3	3	0.006	42.4
2786	3	3	0.033	41.0
2787	3	3	0.000	36.2
2788	3	3	0.003	44.4
2789	3	3	0.004	40.2
2790	4	3	0.002	37.8
2791	4	3	0.000	36.8
2792	4	3	0.044	32.4
2793	4	3	0.000	34.2
2794	4	3	0.020	39.0
2795	4	3	0.003	40.2
2796	4	3	0.008	36.2
2797	4	3	0.000	34.4
2798	4	3	0.000	41.4
2799	4	3	0.000	36.4
2800	4	3	0.015	37.0
2801	4	3	0.010	33.2
2802	4	3	0.000	36.4
2803	4	3	0.000	40.6
2804	4	3	0.000	36.6
2805	4	3	0.000	41.0
2806	4	3	0.003	35.4
2807	4	3	0.000	38.2
2808	4	3	0.006	44.4
2809	4	3	0.006	35.4
2810	4	3	0.000	38.6
2811	4	3	0.002	40.2
2812	4	3	0.000	43.4
2813	4	3	0.000	37.4
2814	4	3	0.000	34.0
2815	4	3	0.007	31.0
2816	4	3	0.000	47.0
2817	4	3	0.016	39.2
2818	4	3	0.018	40.0
2819	4	3	0.012	33.0
2700	1	6	0.000	51.5
2701	1	6	0.000	49.4
2702	1	6	0.047	44.0
2703	1	6	0.001	47.6
2704	1	6	0.000	63.0
2705	1	6	0.004	49.0
2706	1	6	0.000	42.4
2707	1	6	0.009	39.6
2708	1	6	0.040	43.6
2709	1	6	0.016	49.2
2710	1	6	0.012	41.8
2711	1	6	0.033	35.8
2712	1	6	0.019	39.4
2713	1	6	0.001	32.8
2714	1	6	0.000	40.4
2715	1	6	0.002	40.8
2716	1	6	0.000	37.8
2717	1	6	0.000	44.8
2718	1	6	0.000	48.2
2719	1	6	0.003	50.0
2720	1	6	0.000	64.0
2721	1	6	0.000	41.2
2722	1	6	0.000	45.8
2723	1	6	0.000	42.0
2724	1	6	0.007	48.0
2725	1	6	0.000	51.5
2726	1	6	0.000	47.0
2727	1	6	0.000	59.0
2728	1	6	0.000	46.6
2729	1	6	0.001	43.2
2730	2	6	0.018	41.8
2731	2	6	0.001	58.0
2732	2	6	.	.
2733	2	6	0.008	51.5
2734	2	6	0.000	62.5
2735	2	6	0.000	35.4
2736	2	6	0.000	45.0
2737	2	6	0.000	50.0
2738	2	6	0.000	35.0
2739	2	6	0.002	46.6
2740	2	6	0.000	61.0

2741	2	6	0.000	50.5
2742	2	6	0.000	49.8
2743	2	6	0.000	48.6
2744	2	6	0.000	32.0
2745	2	6	0.000	52.0
2746	2	6	0.000	53.5
2747	2	6	0.000	53.5
2748	2	6	.	.
2749	2	6	0.000	46.6
2750	2	6	0.002	46.0
2751	2	6	0.000	45.0
2752	2	6	0.005	47.6
2753	2	6	0.010	52.5
2754	2	6	0.000	47.8
2755	2	6	0.000	46.0
2756	2	6	0.013	47.4
2757	2	6	0.000	45.0
2758	2	6	0.000	47.4
2759	2	6	0.014	40.8
2760	3	6	0.003	42.0
2761	3	6	0.009	44.8
2762	3	6	0.030	39.8
2763	3	6	0.020	39.2
2764	3	6	0.005	49.0
2765	3	6	0.000	38.2
2766	3	6	0.000	45.4
2767	3	6	0.002	47.8
2768	3	6	0.003	46.8
2769	3	6	0.001	39.2
2770	3	6	0.010	42.0
2771	3	6	0.014	42.8
2772	3	6	0.000	40.4
2773	3	6	0.003	33.4
2774	3	6	0.033	45.2
2775	3	6	0.000	37.8
2776	3	6	0.000	40.3
2777	3	6	0.000	44.4
2778	3	6	0.000	46.0
2779	3	6	0.003	37.0
2780	3	6	0.004	44.2
2781	3	6	0.023	45.8
2782	3	6	.	.
2783	3	6	0.013	38.0
2784	3	6	0.008	43.4
2785	3	6	0.031	48.4
2786	3	6	0.000	42.6
2787	3	6	0.000	36.6
2788	3	6	0.009	46.6
2789	3	6	0.007	47.8
2790	4	6	0.069	42.4
2791	4	6	0.007	44.4
2792	4	6	0.005	35.0
2793	4	6	0.004	33.2
2794	4	6	.	.
2795	4	6	0.000	46.6
2796	4	6	0.007	36.0
2797	4	6	0.019	35.8
2798	4	6	0.004	48.6
2799	4	6	0.000	38.6
2800	4	6	0.011	46.2
2801	4	6	0.017	38.4
2802	4	6	0.000	35.6
2803	4	6	0.000	39.4
2804	4	6	0.000	37.2
2805	4	6	0.000	48.4
2806	4	6	0.000	40.2
2807	4	6	0.000	38.4
2808	4	6	0.012	48.0
2809	4	6	0.000	38.8
2810	4	6	0.000	43.8
2811	4	6	0.000	40.2
2812	4	6	0.001	43.0
2813	4	6	0.000	38.8
2814	4	6	0.007	43.0
2815	4	6	0.000	28.2
2816	4	6	0.000	47.6
2817	4	6	0.008	36.0
2818	4	6	0.071	42.6
2819	4	6	0.005	36.4

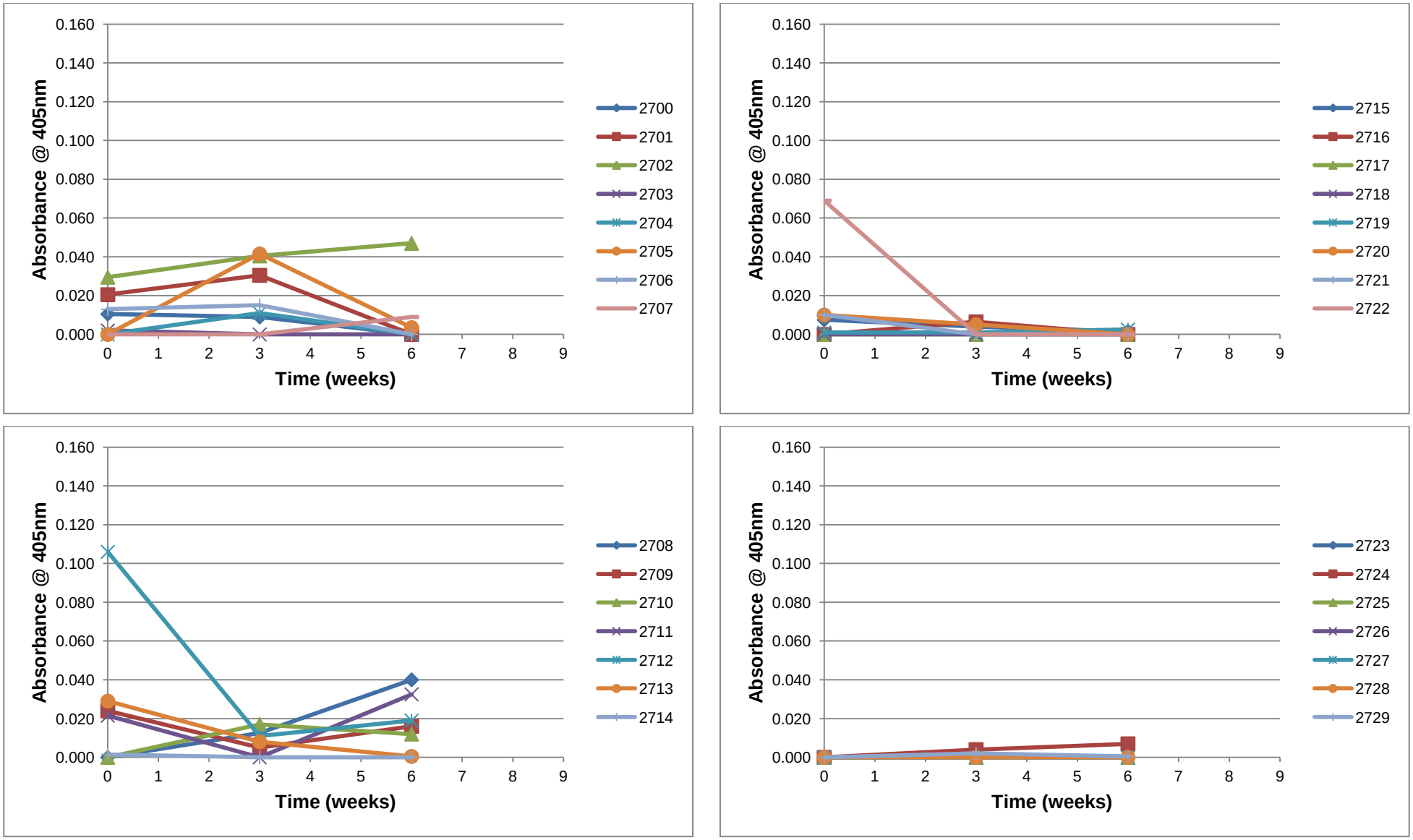

Figure B.9.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of *Salmonella enterica* serovar *typhimurium* SL3261 control

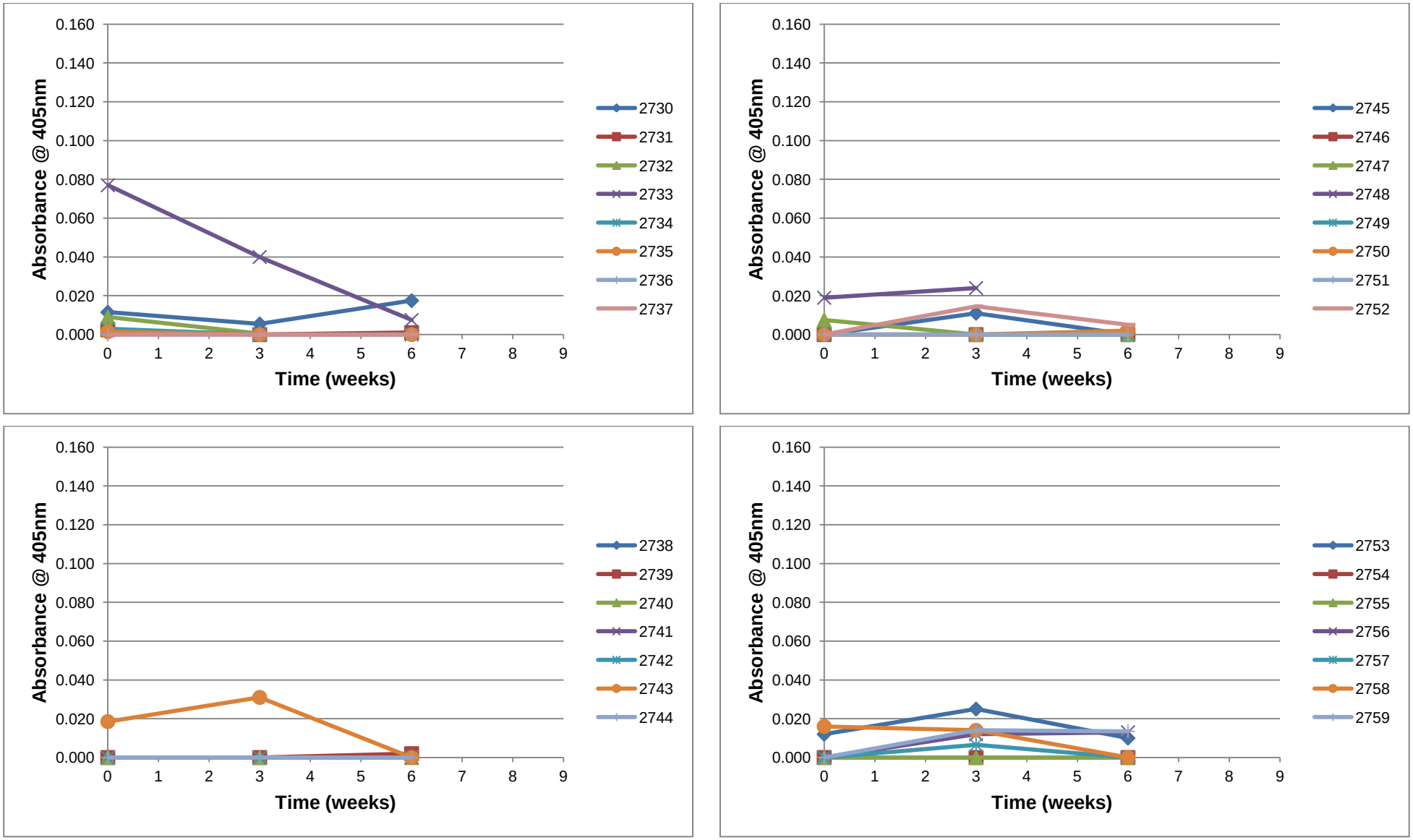

Figure B.10.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of the mucosal VR1020 recombinant vaccine

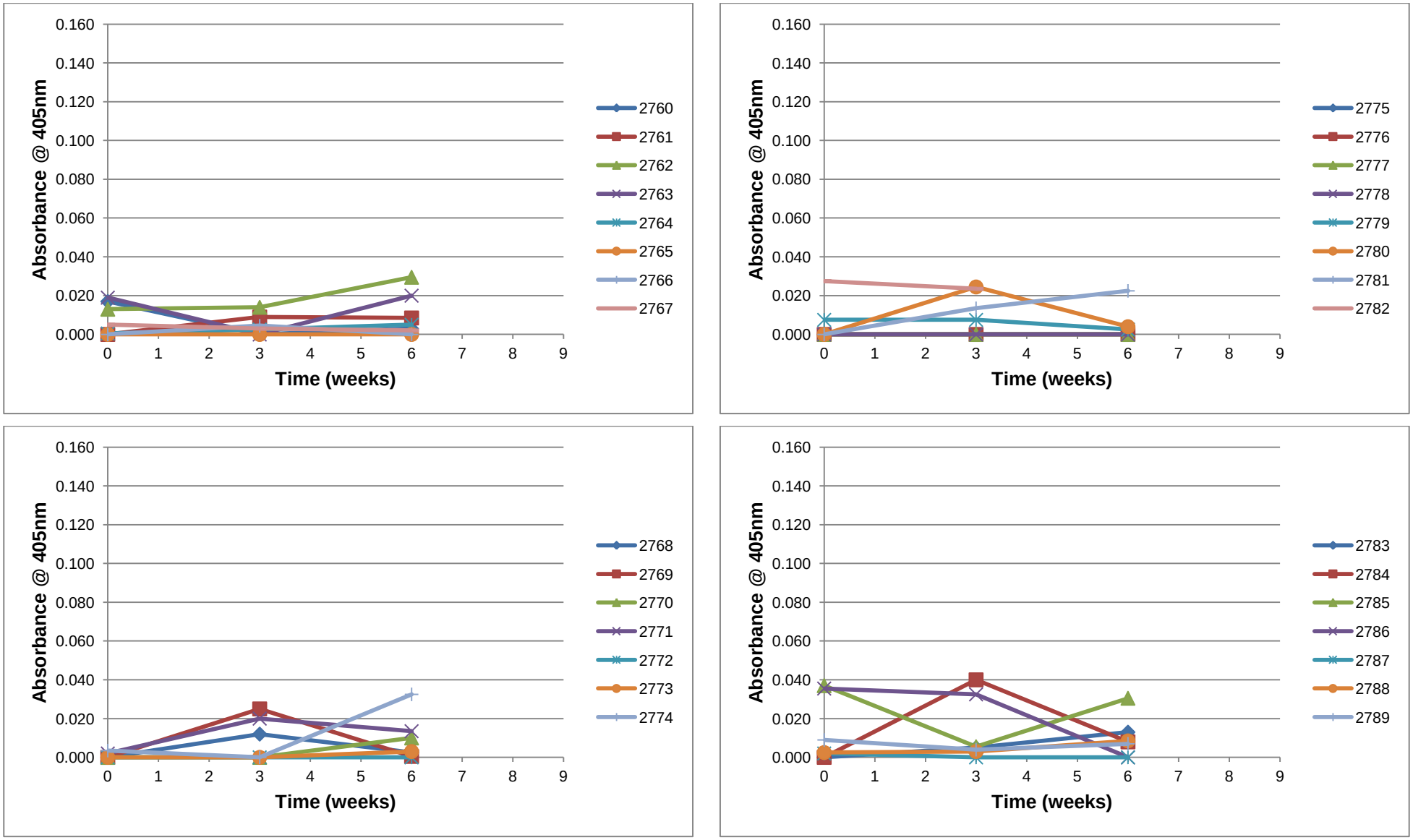

Figure B.11.: Immune responses elicited by ostriches that received 100 µg/ml of the intramuscular VR1020 recombinant vaccine

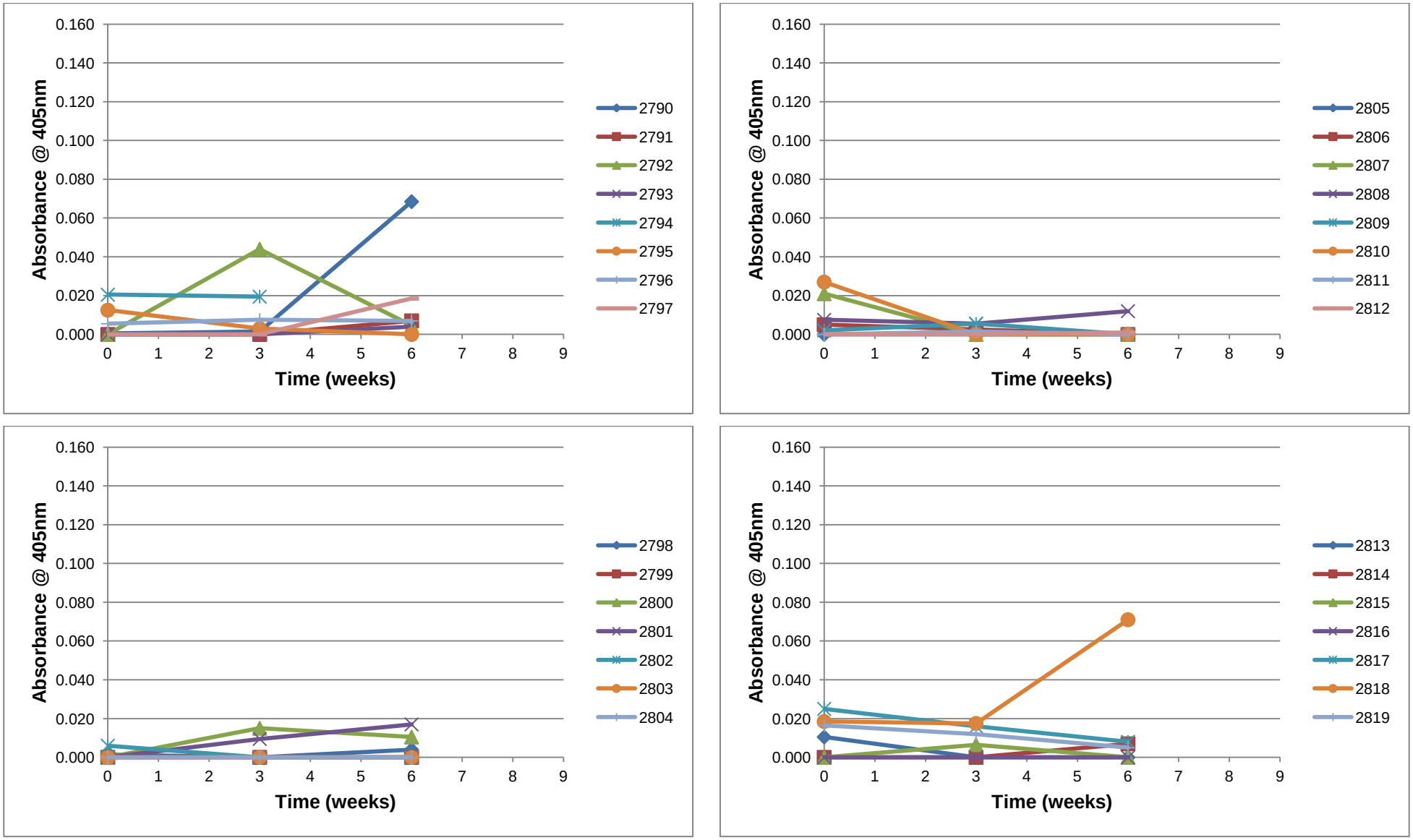

Figure B.12.: Immune responses elicited by ostriches in the control group that received no vaccinations

Humoral immune response of ostriches against *Salmonella entrica* serovar *typhimurium* SL3261 LPS

The humoral immune response of the ostriches against *Salmonella entrica* serovar *typhimurium* SL3261 LPS was evaluated using ELISA as described in chapter as described in chapter 4.2.3.5 and the serum samples collected. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Serum - LPS
Oudsthoorn
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	359	243.951			
Treatment (Treatment)	3	10.061	3.354	5.48	0.0011
Time (Time)	2	19.620	9.810	16.04	0.0000
Treatment x Time	6	1.374	0.229	0.37	0.8951
Residual	348	212.895	0.612		

Grand mean = 1.317 R-squared = 0.1273 C.V. = 59.40%

LSD for Treatment = 0.2293 S.E.D = 0.1166 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.61177

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
1	1.58	54.3	1	Salmonella Control
4	1.31	66.6	2	Control
3	1.25	62.6	3	VR1020 Plasmid
2	1.13	63.9	4	Salmonella + VR1020

LSD for Time = 0.1986 S.E.D = 0.1010 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.61177

Time
Averages

Level	--- Y ---	Cv	Rank	Week
1	1.60	52.6	1	0
2	1.32	58.9	2	3
3	1.03	73.3	3	6

LSD for Treatment*Time = 0.3972 S.E.D = 0.2020 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.61177

Two-way table for Treatment*Time, n=30

	1	2	3
1	1.886	1.624	1.243
2	1.519	1.069	0.798
3	1.468	1.317	0.961
4	1.537	1.260	1.121

The following table the data is arranged in two columns with the ostrich identification number, Treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The Treatments consist of *Salmonella enterica* serovar *typhimurium* SL3261 control (1), mucosal VR1020 recombinant vaccine (2), intramuscular injected VR1020 recombinant vaccine (3) and the control group that received no vaccinations (4). Following the data are the graphs drawn for each ostrich depicting the immune response measured over time.

Ostrich	Trt	Time	Resp	Weight
2700	1	0	2.559	46.6
2701	1	0	2.299	45.0
2702	1	0	2.031	43.0
2703	1	0	1.936	38.0
2704	1	0	2.823	49.0
2705	1	0	1.421	50.0
2706	1	0	2.901	39.0
2707	1	0	2.641	40.0
2708	1	0	1.441	48.0
2709	1	0	0.928	43.0
2710	1	0	0.825	38.0
2711	1	0	2.763	39.0
2712	1	0	0.520	39.0
2713	1	0	0.390	36.0
2714	1	0	0.820	34.0
2715	1	0	2.804	39.0
2716	1	0	2.318	42.0
2717	1	0	1.690	46.0
2718	1	0	1.637	39.0
2719	1	0	2.951	45.0
2720	1	0	1.590	51.0
2721	1	0	0.598	40.0
2722	1	0	2.886	39.0
2723	1	0	1.670	42.0
2724	1	0	2.766	43.0
2725	1	0	1.609	43.0
2726	1	0	1.037	39.0
2727	1	0	0.816	49.0
2728	1	0	3.070	47.0
2729	1	0	2.824	39.8
2730	2	0	2.288	44.1
2731	2	0	2.519	46.0
2732	2	0	0.499	41.0
2733	2	0	1.211	45.0
2734	2	0	1.871	47.0
2735	2	0	0.000	35.0
2736	2	0	0.324	40.0
2737	2	0	1.782	41.0
2738	2	0	2.586	34.0
2739	2	0	2.128	38.0
2740	2	0	1.868	48.0
2741	2	0	0.425	40.0
2742	2	0	1.220	42.0
2743	2	0	2.750	46.0
2744	2	0	1.961	36.0
2745	2	0	1.613	49.0
2746	2	0	1.290	41.0
2747	2	0	1.886	43.0
2748	2	0	1.145	41.0
2749	2	0	1.353	36.0
2750	2	0	1.204	38.0
2751	2	0	1.486	39.0
2752	2	0	0.613	40.0
2753	2	0	2.845	42.0
2754	2	0	2.479	44.0
2755	2	0	0.838	44.0
2756	2	0	0.366	43.0
2757	2	0	1.139	38.0
2758	2	0	2.801	36.0
2759	2	0	1.094	43.0
2760	3	0	0.818	40.0
2761	3	0	0.599	40.0
2762	3	0	1.501	38.0
2763	3	0	2.535	43.0
2764	3	0	0.381	41.0
2765	3	0	0.598	37.0
2766	3	0	1.332	44.0
2767	3	0	1.363	40.0
2768	3	0	0.833	39.0
2769	3	0	1.262	39.0
2770	3	0	1.517	37.0
2771	3	0	3.020	48.0
2772	3	0	2.025	37.0
2773	3	0	1.688	37.0
2774	3	0	1.440	43.0
2775	3	0	1.132	39.0
2776	3	0	2.190	37.0
2777	3	0	1.208	39.0
2778	3	0	2.432	41.0

2779	3	0	1.891	37.0
2780	3	0	2.523	40.0
2781	3	0	3.115	39.0
2782	3	0	0.486	39.0
2783	3	0	0.358	40.0
2784	3	0	1.784	41.0
2785	3	0	1.649	41.0
2786	3	0	0.722	41.0
2787	3	0	0.832	39.0
2788	3	0	0.583	39.0
2789	3	0	2.206	39.0
2790	4	0	2.180	38.0
2791	4	0	1.867	40.0
2792	4	0	1.620	36.0
2793	4	0	1.086	38.0
2794	4	0	1.289	39.0
2795	4	0	0.865	37.0
2796	4	0	1.213	40.0
2797	4	0	2.512	37.0
2798	4	0	0.604	36.0
2799	4	0	1.913	36.0
2800	4	0	2.682	37.0
2801	4	0	1.166	37.0
2802	4	0	3.171	36.0
2803	4	0	0.776	37.0
2804	4	0	2.901	35.0
2805	4	0	0.722	43.0
2806	4	0	2.091	38.0
2807	4	0	1.418	38.0
2808	4	0	2.868	45.0
2809	4	0	2.733	37.0
2810	4	0	1.731	38.0
2811	4	0	0.643	38.0
2812	4	0	0.748	40.0
2813	4	0	2.351	38.0
2814	4	0	1.182	37.0
2815	4	0	2.450	36.0
2816	4	0	0.381	42.0
2817	4	0	0.284	37.0
2818	4	0	0.474	36.0
2819	4	0	0.201	35.0
2700	1	3	2.351	50.5
2701	1	3	1.892	45.4
2702	1	3	1.665	43.0
2703	1	3	1.335	40.6
2704	1	3	2.637	55.5
2705	1	3	1.604	46.0
2706	1	3	2.419	39.2
2707	1	3	1.888	37.8
2708	1	3	0.557	42.0
2709	1	3	0.634	44.8

2710	1	3	0.508	40.2
2711	1	3	1.114	35.2
2712	1	3	0.662	37.2
2713	1	3	0.169	33.6
2714	1	3	1.213	34.4
2715	1	3	0.883	35.0
2716	1	3	1.057	35.6
2717	1	3	1.370	39.8
2718	1	3	1.748	43.8
2719	1	3	3.062	44.6
2720	1	3	0.951	56.5
2721	1	3	1.290	39.2
2722	1	3	2.905	43.6
2723	1	3	1.470	40.2
2724	1	3	1.462	42.8
2725	1	3	1.796	45.4
2726	1	3	2.415	40.8
2727	1	3	1.891	49.6
2728	1	3	3.009	44.4
2729	1	3	2.774	41.6
2730	2	3	1.020	43.2
2731	2	3	2.201	47.4
2732	2	3	0.489	42.8
2733	2	3	0.588	46.8
2734	2	3	1.121	52.0
2735	2	3	0.831	30.4
2736	2	3	0.648	38.8
2737	2	3	2.089	46.4
2738	2	3	0.740	36.4
2739	2	3	1.681	38.2
2740	2	3	0.912	49.2
2741	2	3	0.206	43.8
2742	2	3	0.939	42.4
2743	2	3	1.893	44.8
2744	2	3	1.307	34.8
2745	2	3	0.470	50.5
2746	2	3	0.464	41.2
2747	2	3	0.990	44.4
2748	2	3	2.002	36.8
2749	2	3	0.319	39.4
2750	2	3	0.687	40.2
2751	2	3	1.730	34.8
2752	2	3	0.949	39.0
2753	2	3	0.864	41.6
2754	2	3	1.793	40.6
2755	2	3	0.696	44.4
2756	2	3	0.636	45.2
2757	2	3	0.554	40.6
2758	2	3	2.405	40.2
2759	2	3	0.847	41.0
2760	3	3	0.768	39.4

2761	3	3	0.573	42.6
2762	3	3	1.092	37.0
2763	3	3	2.178	39.8
2764	3	3	0.626	41.2
2765	3	3	0.810	37.8
2766	3	3	1.064	40.6
2767	3	3	1.337	43.2
2768	3	3	0.817	43.2
2769	3	3	1.246	38.8
2770	3	3	1.360	43.8
2771	3	3	2.960	41.4
2772	3	3	2.800	40.8
2773	3	3	1.922	37.6
2774	3	3	0.783	46.6
2775	3	3	1.935	32.0
2776	3	3	1.553	37.8
2777	3	3	1.085	41.4
2778	3	3	1.000	44.6
2779	3	3	1.909	38.0
2780	3	3	2.045	41.8
2781	3	3	2.921	39.2
2782	3	3	0.368	37.6
2783	3	3	0.230	35.0
2784	3	3	0.677	40.8
2785	3	3	1.078	42.4
2786	3	3	0.526	41.0
2787	3	3	1.530	36.2
2788	3	3	0.966	44.4
2789	3	3	1.321	40.2
2790	4	3	1.772	37.8
2791	4	3	0.736	36.8
2792	4	3	0.463	32.4
2793	4	3	1.324	34.2
2794	4	3	0.801	39.0
2795	4	3	1.424	40.2
2796	4	3	0.000	36.2
2797	4	3	2.090	34.4
2798	4	3	0.553	41.4
2799	4	3	1.160	36.4
2800	4	3	2.466	37.0
2801	4	3	0.889	33.2
2802	4	3	2.860	36.4
2803	4	3	0.726	40.6
2804	4	3	2.614	36.6
2805	4	3	1.004	41.0
2806	4	3	1.441	35.4
2807	4	3	1.339	38.2
2808	4	3	2.893	44.4
2809	4	3	2.492	35.4
2810	4	3	0.617	38.6
2811	4	3	1.497	40.2

2812	4	3	0.787	43.4
2813	4	3	1.870	37.4
2814	4	3	0.299	34.0
2815	4	3	2.489	31.0
2816	4	3	0.122	47.0
2817	4	3	0.376	39.2
2818	4	3	0.374	40.0
2819	4	3	0.339	33.0
2700	1	6	2.100	51.5
2701	1	6	1.775	49.4
2702	1	6	1.355	44.0
2703	1	6	1.575	47.6
2704	1	6	1.768	63.0
2705	1	6	1.494	49.0
2706	1	6	2.007	42.4
2707	1	6	1.346	39.6
2708	1	6	0.835	43.6
2709	1	6	0.805	49.2
2710	1	6	0.812	41.8
2711	1	6	1.983	35.8
2712	1	6	0.705	39.4
2713	1	6	0.044	32.8
2714	1	6	0.639	40.4
2715	1	6	0.362	40.8
2716	1	6	0.496	37.8
2717	1	6	0.941	44.8
2718	1	6	2.707	48.2
2719	1	6	2.776	50.0
2720	1	6	0.350	64.0
2721	1	6	0.468	41.2
2722	1	6	0.696	45.8
2723	1	6	1.107	42.0
2724	1	6	0.387	48.0
2725	1	6	1.240	51.5
2726	1	6	0.380	47.0
2727	1	6	0.536	59.0
2728	1	6	2.769	46.6
2729	1	6	2.781	43.2
2730	2	6	0.738	41.8
2731	2	6	0.770	58.0
2732	2	6	.	.
2733	2	6	0.856	51.5
2734	2	6	0.280	62.5
2735	2	6	0.856	35.4
2736	2	6	0.423	45.0
2737	2	6	1.411	50.0
2738	2	6	0.328	35.0
2739	2	6	0.934	46.6
2740	2	6	0.474	61.0
2741	2	6	2.392	50.5
2742	2	6	0.732	49.8

2743	2	6	0.681	48.6
2744	2	6	0.566	32.0
2745	2	6	0.420	52.0
2746	2	6	0.662	53.5
2747	2	6	0.980	53.5
2748	2	6	.	.
2749	2	6	0.373	46.6
2750	2	6	0.219	46.0
2751	2	6	1.161	45.0
2752	2	6	0.611	47.6
2753	2	6	1.091	52.5
2754	2	6	0.983	47.8
2755	2	6	0.451	46.0
2756	2	6	1.541	47.4
2757	2	6	2.080	45.0
2758	2	6	1.052	47.4
2759	2	6	0.888	40.8
2760	3	6	0.862	42.0
2761	3	6	0.575	44.8
2762	3	6	0.373	39.8
2763	3	6	1.753	39.2
2764	3	6	0.328	49.0
2765	3	6	0.767	38.2
2766	3	6	0.578	45.4
2767	3	6	0.817	47.8
2768	3	6	0.681	46.8
2769	3	6	0.473	39.2
2770	3	6	0.703	42.0
2771	3	6	2.823	42.8
2772	3	6	2.148	40.4
2773	3	6	0.653	33.4
2774	3	6	0.250	45.2
2775	3	6	0.557	37.8
2776	3	6	0.556	40.3
2777	3	6	0.511	44.4
2778	3	6	0.769	46.0
2779	3	6	1.410	37.0
2780	3	6	1.083	44.2
2781	3	6	3.094	45.8
2782	3	6	.	.
2783	3	6	2.247	38.0
2784	3	6	0.435	43.4
2785	3	6	0.950	48.4
2786	3	6	0.565	42.6
2787	3	6	1.681	36.6
2788	3	6	0.287	46.6
2789	3	6	0.889	47.8
2790	4	6	1.052	42.4
2791	4	6	0.441	44.4
2792	4	6	0.484	35.0
2793	4	6	0.732	33.2
2794	4	6	.	.
2795	4	6	0.790	46.6
2796	4	6	0.842	36.0
2797	4	6	1.686	35.8
2798	4	6	0.596	48.6
2799	4	6	1.258	38.6
2800	4	6	1.297	46.2
2801	4	6	0.513	38.4
2802	4	6	2.962	35.6
2803	4	6	1.477	39.4
2804	4	6	2.806	37.2
2805	4	6	0.952	48.4
2806	4	6	1.355	40.2
2807	4	6	0.955	38.4
2808	4	6	2.992	48.0
2809	4	6	1.210	38.8
2810	4	6	0.540	43.8
2811	4	6	1.317	40.2
2812	4	6	1.579	43.0
2813	4	6	2.783	38.8
2814	4	6	0.842	43.0
2815	4	6	0.972	28.2
2816	4	6	0.174	47.6
2817	4	6	0.611	36.0
2818	4	6	0.237	42.6
2819	4	6	0.184	36.4

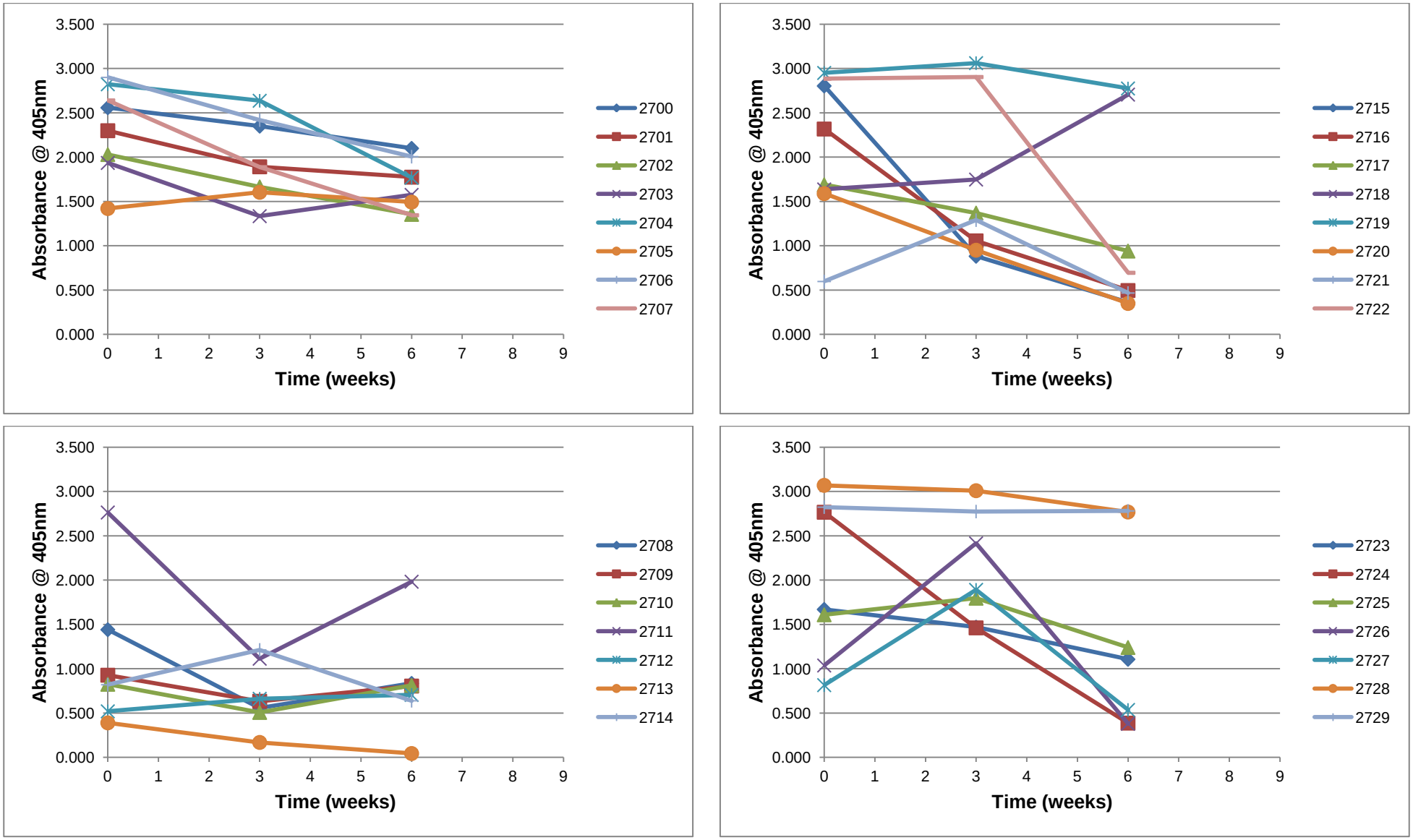

Figure B.13.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of *Salmonella enterica* serovar *typhimurium* SL3261 control

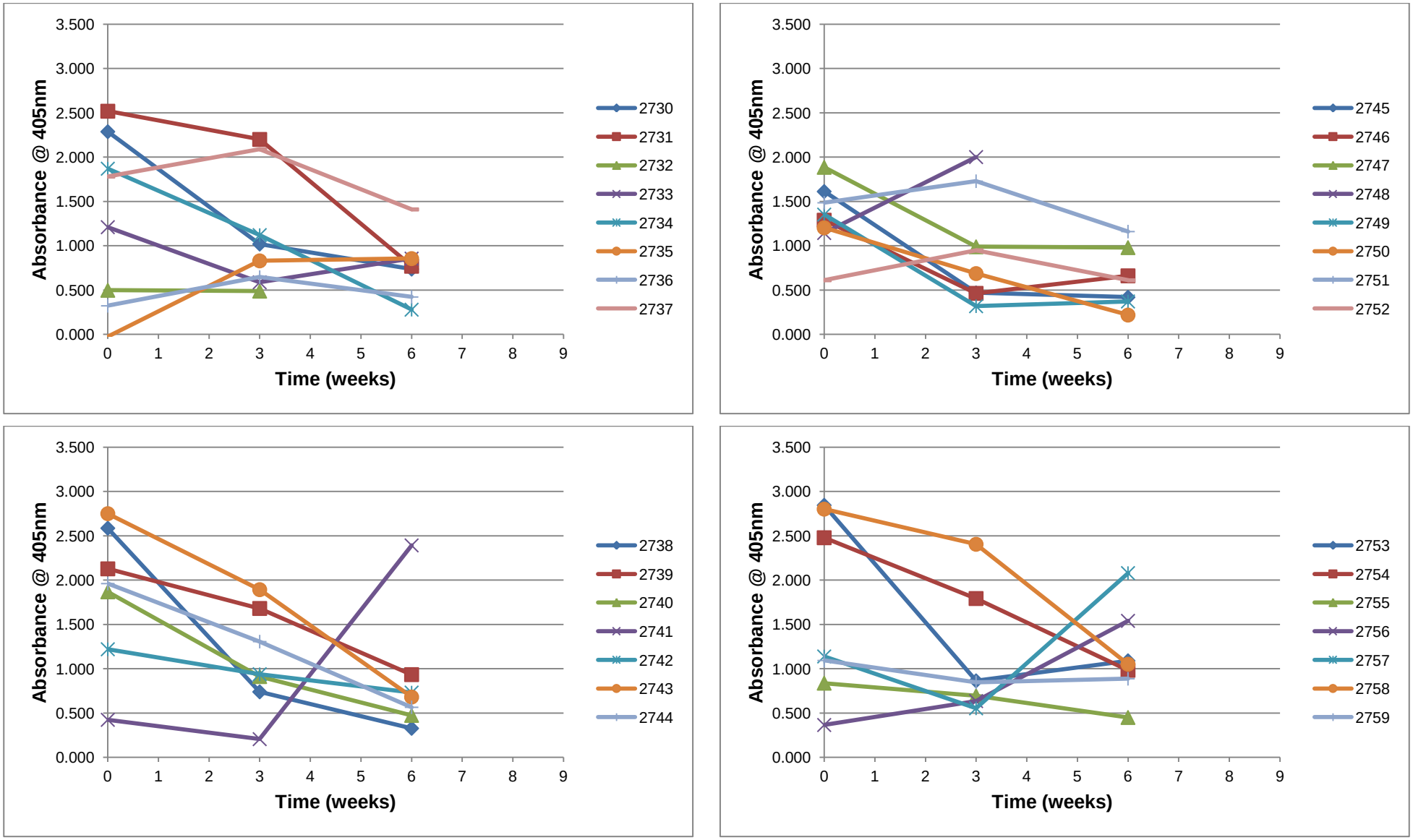

Figure B.14.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of the mucosal VR1020 recombinant vaccine

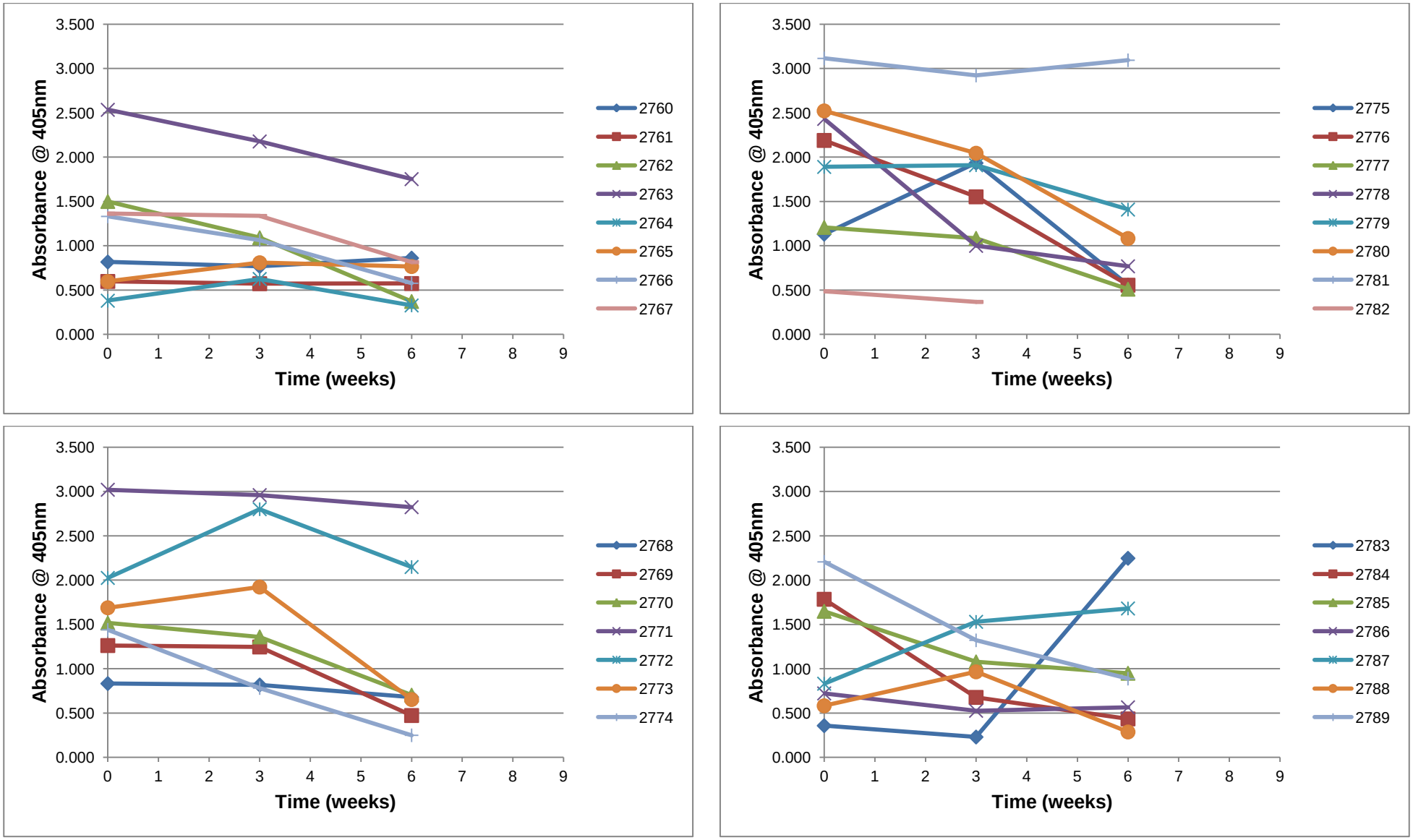

Figure B.15: Immune responses elicited by ostriches that received 100 µg/ml of the intramuscular VR1020 recombinant vaccine

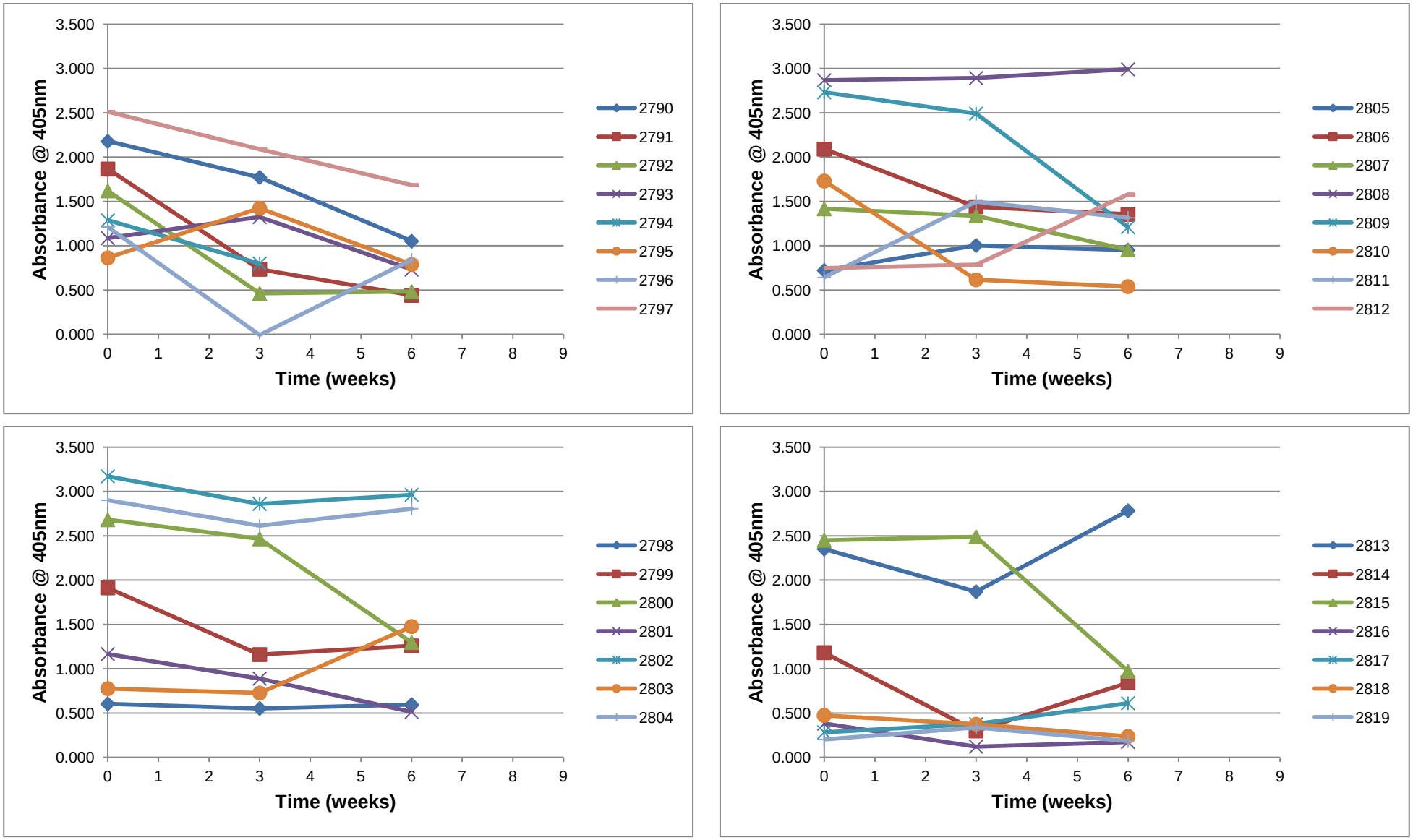

Figure B.16.: Immune responses elicited by ostriches in the control group that received no vaccinations

Mucosal immune response of ostriches against *Salmonella entrica* serovar *typhimurium* SL3261 LPS

The mucosal immune response of the ostriches against *Salmonella entrica* serovar *typhimurium* SL3261 LPS was evaluated using ELISA as described in chapter 4.2.3.5 using the swab samples collected and rabbit anti-ostrich IgA protein 1 as the biotinylated secondary antibody. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – LPS – IgA Protein 1
Oudsthoorn
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	359	0.068			
Treatment (Treatment)	3	0.003	0.001	5.89	0.0006
Time (Time)	2	0.001	0.000	1.49	0.2275
Treatment x Time	6	0.001	0.000	0.54	0.7814
Residual	348	0.063	0.000		

Grand mean = 0.008 R-squared = 0.0642 C.V. = 170.5%

LSD for Treatment = 0.0040 S.E.D = 0.0020 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00018

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
3	0.01	132.3	1	VR1020 Plasmid
1	0.01	201.0	2	Salmonella Control
2	0.01	152.6	3	Salmonella + VR1020
4	0.00	207.9	4	Control

LSD for Time = 0.0034 S.E.D = 0.0017 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00018

Time
Averages

Level	--- Y ---	Cv	Rank	Week
2	0.01	184.2	1	3
3	0.01	167.1	2	6
1	0.01	148.2	3	0

LSD for Treatment*Time = 0.0069 S.E.D = 0.0035 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00018

Two-way table for Treatment*Time, n=30

	1	2	3
1	0.006	0.011	0.007
2	0.005	0.010	0.009
3	0.010	0.011	0.015
4	0.003	0.004	0.003

The following table the data is arranged in two columns with the ostrich identification number, Treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The Treatments consist of *Salmonella enterica* serovar *typhimurium* SL3261 control (1), mucosal VR1020 recombinant vaccine (2), intramuscular injected VR1020 recombinant vaccine (3) and the control group that received no vaccinations (4). Following the data are the graphs drawn for each ostrich depicting the immune response measured over time.

Ostrich	Trt	Time	Resp	Weight
2700	1	0	0.000	46.6
2701	1	0	0.011	45.0
2702	1	0	0.006	43.0
2703	1	0	0.006	38.0
2704	1	0	0.000	49.0
2705	1	0	0.003	50.0
2706	1	0	0.000	39.0
2707	1	0	0.000	40.0
2708	1	0	0.019	48.0
2709	1	0	0.022	43.0
2710	1	0	0.012	38.0
2711	1	0	0.000	39.0
2712	1	0	0.000	39.0
2713	1	0	0.000	36.0
2714	1	0	0.004	34.0
2715	1	0	0.010	39.0
2716	1	0	0.009	42.0
2717	1	0	0.005	46.0
2718	1	0	0.001	39.0
2719	1	0	0.007	45.0
2720	1	0	0.025	51.0
2721	1	0	0.013	40.0
2722	1	0	0.000	39.0
2723	1	0	0.005	42.0
2724	1	0	0.000	43.0
2725	1	0	0.001	43.0
2726	1	0	0.009	39.0
2727	1	0	0.014	49.0
2728	1	0	0.002	47.0
2729	1	0	0.002	39.8
2730	2	0	0.009	44.1
2731	2	0	0.004	46.0
2732	2	0	0.021	41.0
2733	2	0	0.000	45.0
2734	2	0	0.012	47.0
2735	2	0	0.013	35.0
2736	2	0	0.000	40.0
2737	2	0	0.000	41.0
2738	2	0	0.000	34.0
2739	2	0	0.000	38.0
2740	2	0	0.000	48.0
2741	2	0	0.000	40.0
2742	2	0	0.000	42.0
2743	2	0	0.000	46.0
2744	2	0	0.001	36.0
2745	2	0	0.018	49.0
2746	2	0	0.010	41.0
2747	2	0	0.000	43.0
2748	2	0	0.006	41.0
2749	2	0	0.013	36.0
2750	2	0	0.010	38.0
2751	2	0	0.000	39.0
2752	2	0	0.000	40.0
2753	2	0	0.001	42.0
2754	2	0	0.000	44.0
2755	2	0	0.000	44.0
2756	2	0	0.022	43.0
2757	2	0	0.026	38.0
2758	2	0	0.000	36.0
2759	2	0	0.000	43.0
2760	3	0	0.000	40.0
2761	3	0	0.000	40.0
2762	3	0	0.000	38.0
2763	3	0	0.016	43.0
2764	3	0	0.032	41.0
2765	3	0	0.000	37.0
2766	3	0	0.000	44.0
2767	3	0	0.006	40.0
2768	3	0	0.001	39.0
2769	3	0	0.000	39.0
2770	3	0	0.000	37.0
2771	3	0	0.000	48.0
2772	3	0	0.015	37.0
2773	3	0	0.006	37.0
2774	3	0	0.004	43.0
2775	3	0	0.006	39.0
2776	3	0	0.020	37.0
2777	3	0	0.022	39.0
2778	3	0	0.001	41.0

2779	3	0	0.006	37.0
2780	3	0	0.026	40.0
2781	3	0	0.034	39.0
2782	3	0	0.000	39.0
2783	3	0	0.039	40.0
2784	3	0	0.000	41.0
2785	3	0	0.013	41.0
2786	3	0	0.042	41.0
2787	3	0	0.000	39.0
2788	3	0	0.010	39.0
2789	3	0	0.000	39.0
2790	4	0	0.000	38.0
2791	4	0	0.006	40.0
2792	4	0	0.000	36.0
2793	4	0	0.000	38.0
2794	4	0	0.003	39.0
2795	4	0	0.012	37.0
2796	4	0	0.000	40.0
2797	4	0	0.008	37.0
2798	4	0	0.000	36.0
2799	4	0	0.003	36.0
2800	4	0	0.000	37.0
2801	4	0	0.000	37.0
2802	4	0	0.011	36.0
2803	4	0	0.003	37.0
2804	4	0	0.000	35.0
2805	4	0	0.000	43.0
2806	4	0	0.016	38.0
2807	4	0	0.009	38.0
2808	4	0	0.016	45.0
2809	4	0	0.000	37.0
2810	4	0	0.000	38.0
2811	4	0	0.000	38.0
2812	4	0	0.000	40.0
2813	4	0	0.000	38.0
2814	4	0	0.000	37.0
2815	4	0	0.011	36.0
2816	4	0	0.005	42.0
2817	4	0	0.000	37.0
2818	4	0	0.000	36.0
2819	4	0	0.000	35.0
2700	1	3	0.000	50.5
2701	1	3	0.007	45.4
2702	1	3	0.000	43.0
2703	1	3	0.017	40.6
2704	1	3	0.005	55.5
2705	1	3	0.000	46.0
2706	1	3	0.000	39.2
2707	1	3	0.000	37.8
2708	1	3	0.008	42.0
2709	1	3	0.143	44.8

2710	1	3	0.028	40.2
2711	1	3	0.000	35.2
2712	1	3	0.000	37.2
2713	1	3	0.000	33.6
2714	1	3	0.003	34.4
2715	1	3	0.009	35.0
2716	1	3	0.006	35.6
2717	1	3	0.012	39.8
2718	1	3	0.004	43.8
2719	1	3	0.009	44.6
2720	1	3	0.020	56.5
2721	1	3	0.005	39.2
2722	1	3	0.002	43.6
2723	1	3	0.000	40.2
2724	1	3	0.003	42.8
2725	1	3	0.001	45.4
2726	1	3	0.016	40.8
2727	1	3	0.007	49.6
2728	1	3	0.000	44.4
2729	1	3	0.022	41.6
2730	2	3	0.039	43.2
2731	2	3	0.026	47.4
2732	2	3	0.018	42.8
2733	2	3	0.000	46.8
2734	2	3	0.013	52.0
2735	2	3	0.038	30.4
2736	2	3	0.005	38.8
2737	2	3	0.000	46.4
2738	2	3	0.000	36.4
2739	2	3	0.000	38.2
2740	2	3	0.000	49.2
2741	2	3	0.000	43.8
2742	2	3	0.000	42.4
2743	2	3	0.001	44.8
2744	2	3	0.026	34.8
2745	2	3	0.014	50.5
2746	2	3	0.011	41.2
2747	2	3	0.000	44.4
2748	2	3	0.011	36.8
2749	2	3	0.003	39.4
2750	2	3	0.048	40.2
2751	2	3	0.000	34.8
2752	2	3	0.009	39.0
2753	2	3	0.003	41.6
2754	2	3	0.000	40.6
2755	2	3	0.010	44.4
2756	2	3	0.005	45.2
2757	2	3	0.022	40.6
2758	2	3	0.000	40.2
2759	2	3	0.000	41.0
2760	3	3	0.000	39.4

2761	3	3	0.014	42.6
2762	3	3	0.000	37.0
2763	3	3	0.022	39.8
2764	3	3	0.018	41.2
2765	3	3	0.000	37.8
2766	3	3	0.000	40.6
2767	3	3	0.000	43.2
2768	3	3	0.005	43.2
2769	3	3	0.000	38.8
2770	3	3	0.000	43.8
2771	3	3	0.015	41.4
2772	3	3	0.017	40.8
2773	3	3	0.012	37.6
2774	3	3	0.009	46.6
2775	3	3	0.012	32.0
2776	3	3	0.042	37.8
2777	3	3	0.004	41.4
2778	3	3	0.011	44.6
2779	3	3	0.000	38.0
2780	3	3	0.026	41.8
2781	3	3	0.040	39.2
2782	3	3	0.011	37.6
2783	3	3	0.010	35.0
2784	3	3	0.000	40.8
2785	3	3	0.050	42.4
2786	3	3	0.028	41.0
2787	3	3	0.000	36.2
2788	3	3	0.005	44.4
2789	3	3	0.000	40.2
2790	4	3	0.000	37.8
2791	4	3	0.000	36.8
2792	4	3	0.000	32.4
2793	4	3	0.000	34.2
2794	4	3	0.006	39.0
2795	4	3	0.001	40.2
2796	4	3	0.000	36.2
2797	4	3	0.000	34.4
2798	4	3	0.000	41.4
2799	4	3	0.002	36.4
2800	4	3	0.001	37.0
2801	4	3	0.000	33.2
2802	4	3	0.022	36.4
2803	4	3	0.009	40.6
2804	4	3	0.002	36.6
2805	4	3	0.000	41.0
2806	4	3	0.005	35.4
2807	4	3	0.020	38.2
2808	4	3	0.013	44.4
2809	4	3	0.000	35.4
2810	4	3	0.007	38.6
2811	4	3	0.000	40.2
2812	4	3	0.021	43.4
2813	4	3	0.000	37.4
2814	4	3	0.000	34.0
2815	4	3	0.029	31.0
2816	4	3	0.000	47.0
2817	4	3	0.000	39.2
2818	4	3	0.000	40.0
2819	4	3	0.000	33.0
2700	1	6	0.000	51.5
2701	1	6	0.000	49.4
2702	1	6	0.001	44.0
2703	1	6	0.000	47.6
2704	1	6	0.000	63.0
2705	1	6	0.000	49.0
2706	1	6	0.004	42.4
2707	1	6	0.000	39.6
2708	1	6	0.000	43.6
2709	1	6	0.018	49.2
2710	1	6	0.037	41.8
2711	1	6	0.000	35.8
2712	1	6	0.000	39.4
2713	1	6	0.000	32.8
2714	1	6	0.008	40.4
2715	1	6	0.010	40.8
2716	1	6	0.006	37.8
2717	1	6	0.002	44.8
2718	1	6	0.011	48.2
2719	1	6	0.014	50.0
2720	1	6	0.006	64.0
2721	1	6	0.000	41.2
2722	1	6	0.018	45.8
2723	1	6	0.003	42.0
2724	1	6	0.025	48.0
2725	1	6	0.030	51.5
2726	1	6	0.009	47.0
2727	1	6	0.004	59.0
2728	1	6	0.000	46.6
2729	1	6	0.012	43.2
2730	2	6	0.009	41.8
2731	2	6	0.035	58.0
2732	2	6	.	.
2733	2	6	0.007	51.5
2734	2	6	0.009	62.5
2735	2	6	0.005	35.4
2736	2	6	0.000	45.0
2737	2	6	0.000	50.0
2738	2	6	0.000	35.0
2739	2	6	0.000	46.6
2740	2	6	0.000	61.0
2741	2	6	0.000	50.5
2742	2	6	0.018	49.8

2743	2	6	0.000	48.6
2744	2	6	0.016	32.0
2745	2	6	0.031	52.0
2746	2	6	0.024	53.5
2747	2	6	0.008	53.5
2748	2	6	.	.
2749	2	6	0.050	46.6
2750	2	6	0.010	46.0
2751	2	6	0.000	45.0
2752	2	6	0.003	47.6
2753	2	6	0.000	52.5
2754	2	6	0.000	47.8
2755	2	6	0.000	46.0
2756	2	6	0.023	47.4
2757	2	6	0.023	45.0
2758	2	6	0.000	47.4
2759	2	6	0.000	40.8
2760	3	6	0.007	42.0
2761	3	6	0.009	44.8
2762	3	6	0.010	39.8
2763	3	6	0.080	39.2
2764	3	6	0.000	49.0
2765	3	6	0.000	38.2
2766	3	6	0.000	45.4
2767	3	6	0.000	47.8
2768	3	6	0.000	46.8
2769	3	6	0.003	39.2
2770	3	6	0.000	42.0
2771	3	6	0.022	42.8
2772	3	6	0.019	40.4
2773	3	6	0.019	33.4
2774	3	6	0.005	45.2
2775	3	6	0.010	37.8
2776	3	6	0.011	40.3
2777	3	6	0.010	44.4
2778	3	6	0.024	46.0
2779	3	6	0.000	37.0
2780	3	6	0.067	44.2
2781	3	6	0.049	45.8
2782	3	6	.	.
2783	3	6	0.019	38.0
2784	3	6	0.030	43.4
2785	3	6	0.017	48.4
2786	3	6	0.011	42.6
2787	3	6	0.011	36.6
2788	3	6	0.002	46.6
2789	3	6	0.000	47.8
2790	4	6	0.000	42.4
2791	4	6	0.000	44.4
2792	4	6	0.000	35.0
2793	4	6	0.000	33.2
2794	4	6	.	.
2795	4	6	0.000	46.6
2796	4	6	0.000	36.0
2797	4	6	0.000	35.8
2798	4	6	0.000	48.6
2799	4	6	0.000	38.6
2800	4	6	0.000	46.2
2801	4	6	0.003	38.4
2802	4	6	0.013	35.6
2803	4	6	0.001	39.4
2804	4	6	0.006	37.2
2805	4	6	0.001	48.4
2806	4	6	0.037	40.2
2807	4	6	0.007	38.4
2808	4	6	0.011	48.0
2809	4	6	0.000	38.8
2810	4	6	0.000	43.8
2811	4	6	0.000	40.2
2812	4	6	0.009	43.0
2813	4	6	0.002	38.8
2814	4	6	0.000	43.0
2815	4	6	0.000	28.2
2816	4	6	0.000	47.6
2817	4	6	0.000	36.0
2818	4	6	0.000	42.6
2819	4	6	0.000	36.4

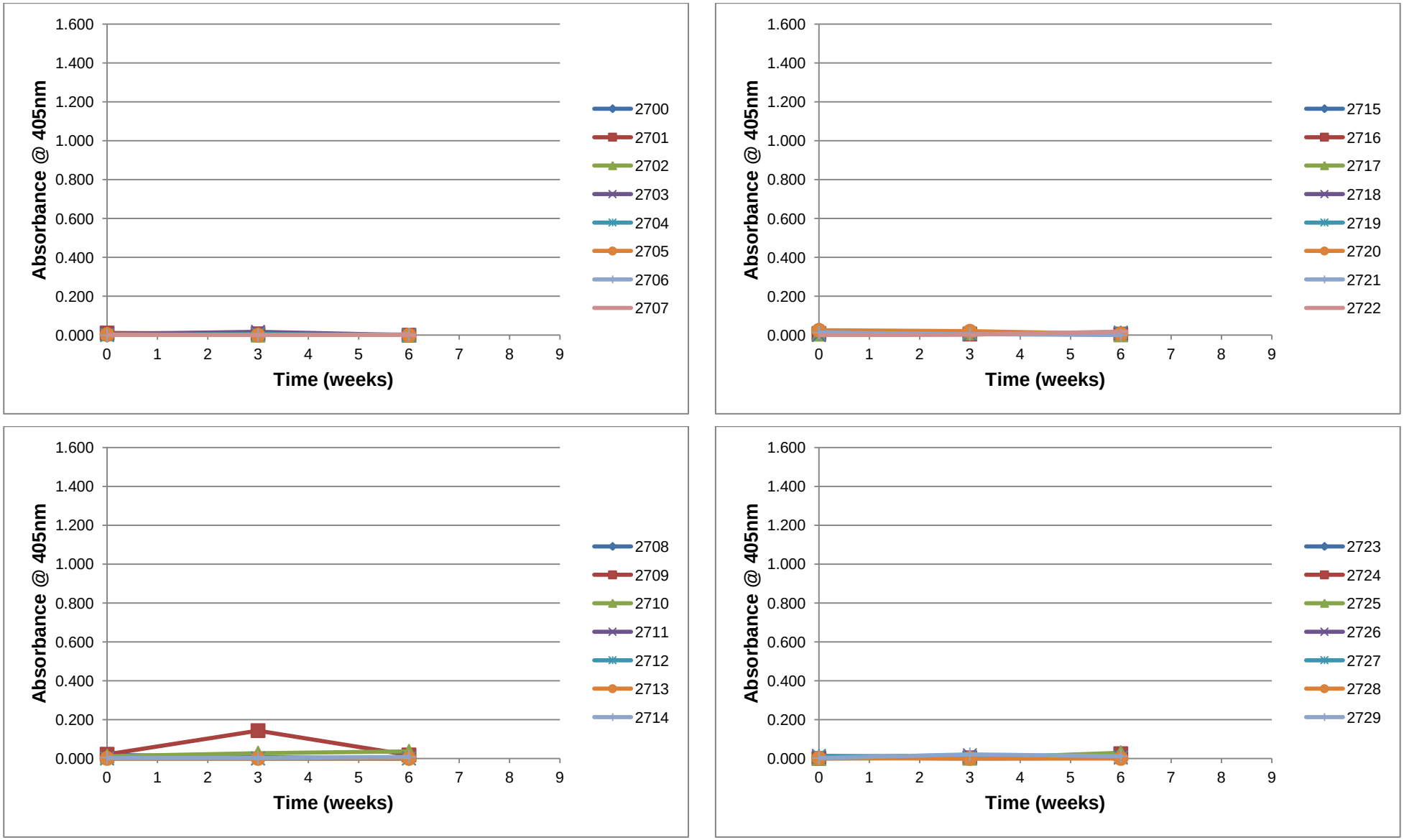

Figure B-17: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of *Salmonella enterica* serovar *typhimurium* SL3261 control

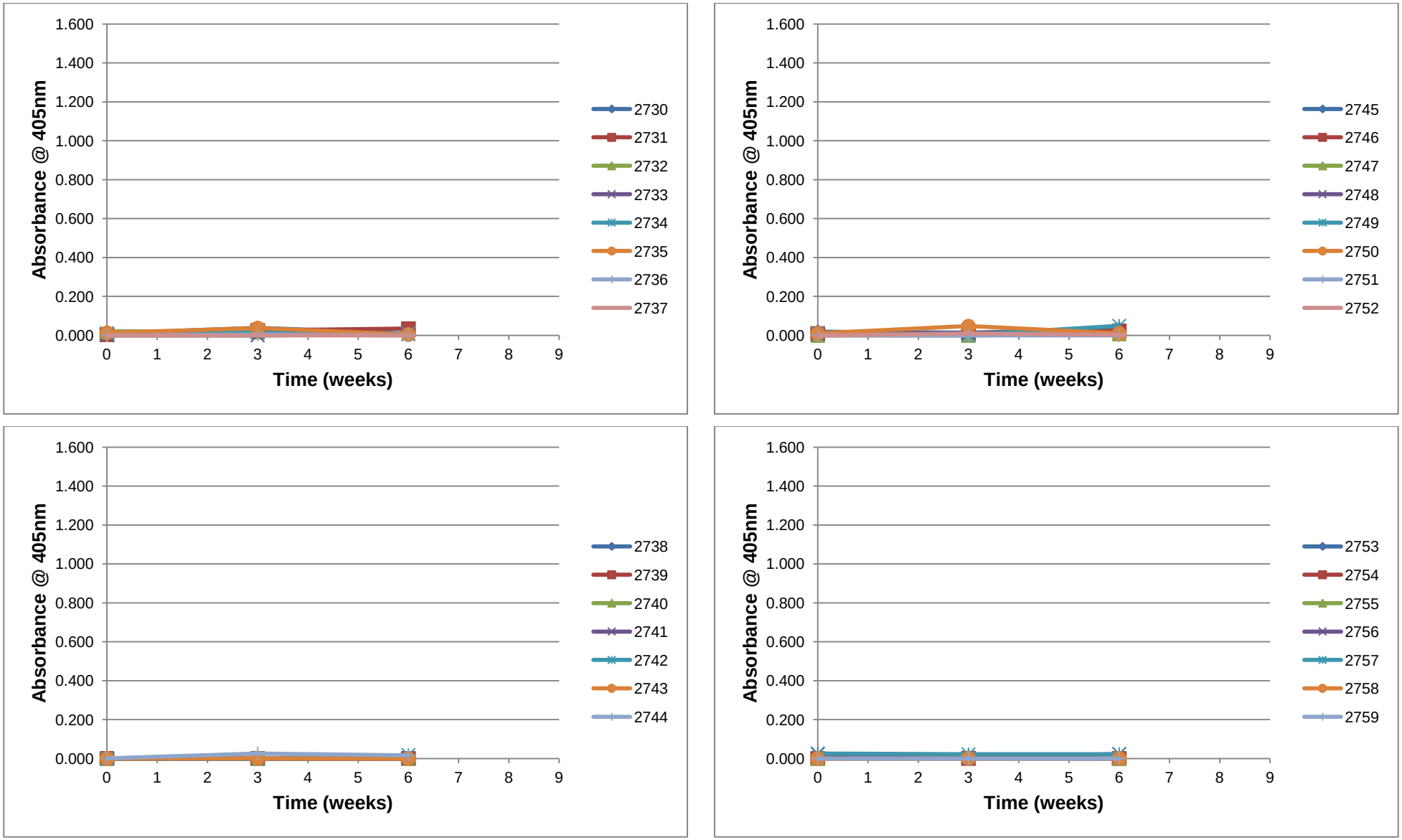

Figure B.18.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of the mucosal VR1020 recombinant vaccine

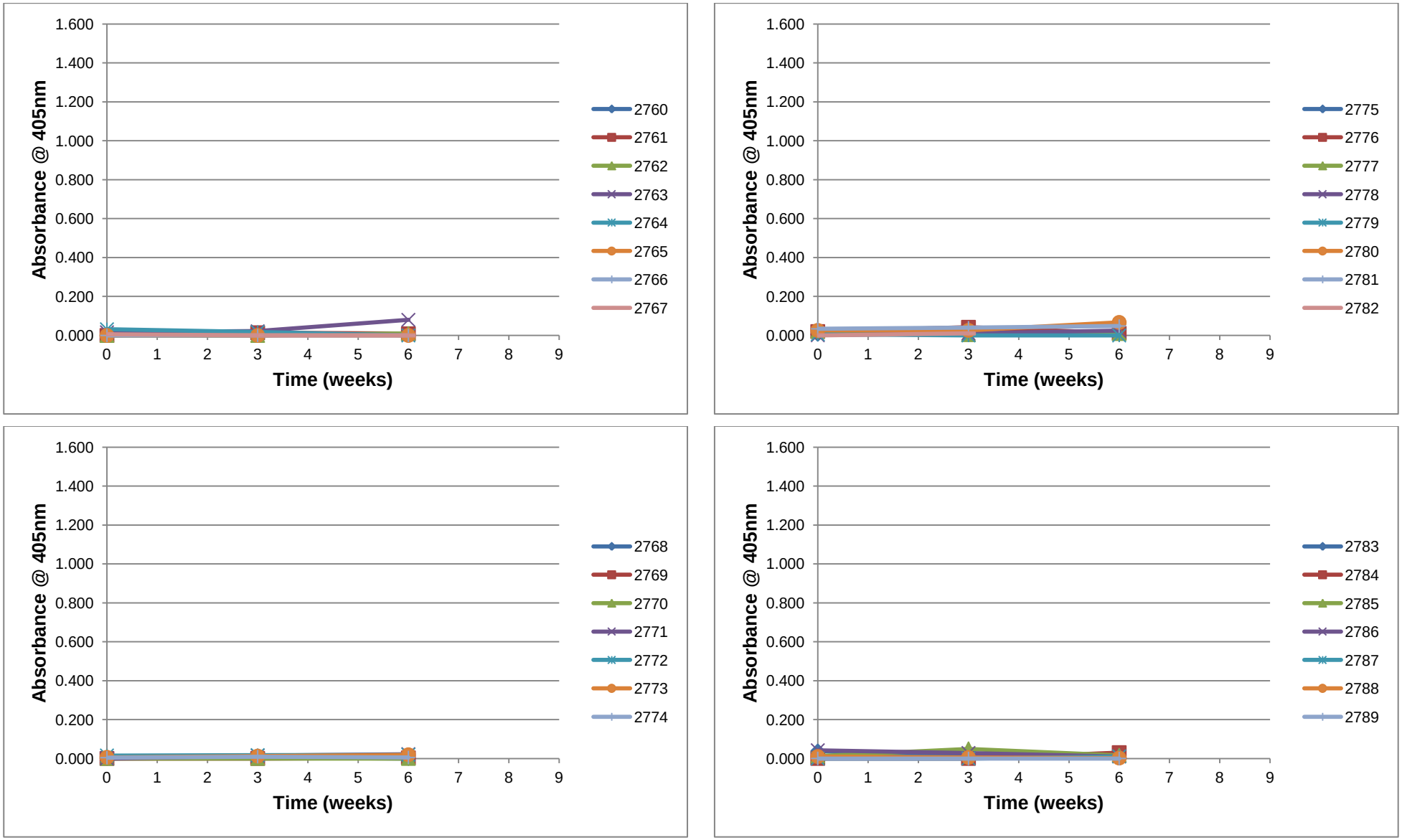

Figure B.19.: Immune responses elicited by ostriches that received 100 µg/ml of the intramuscular VR1020 recombinant vaccine

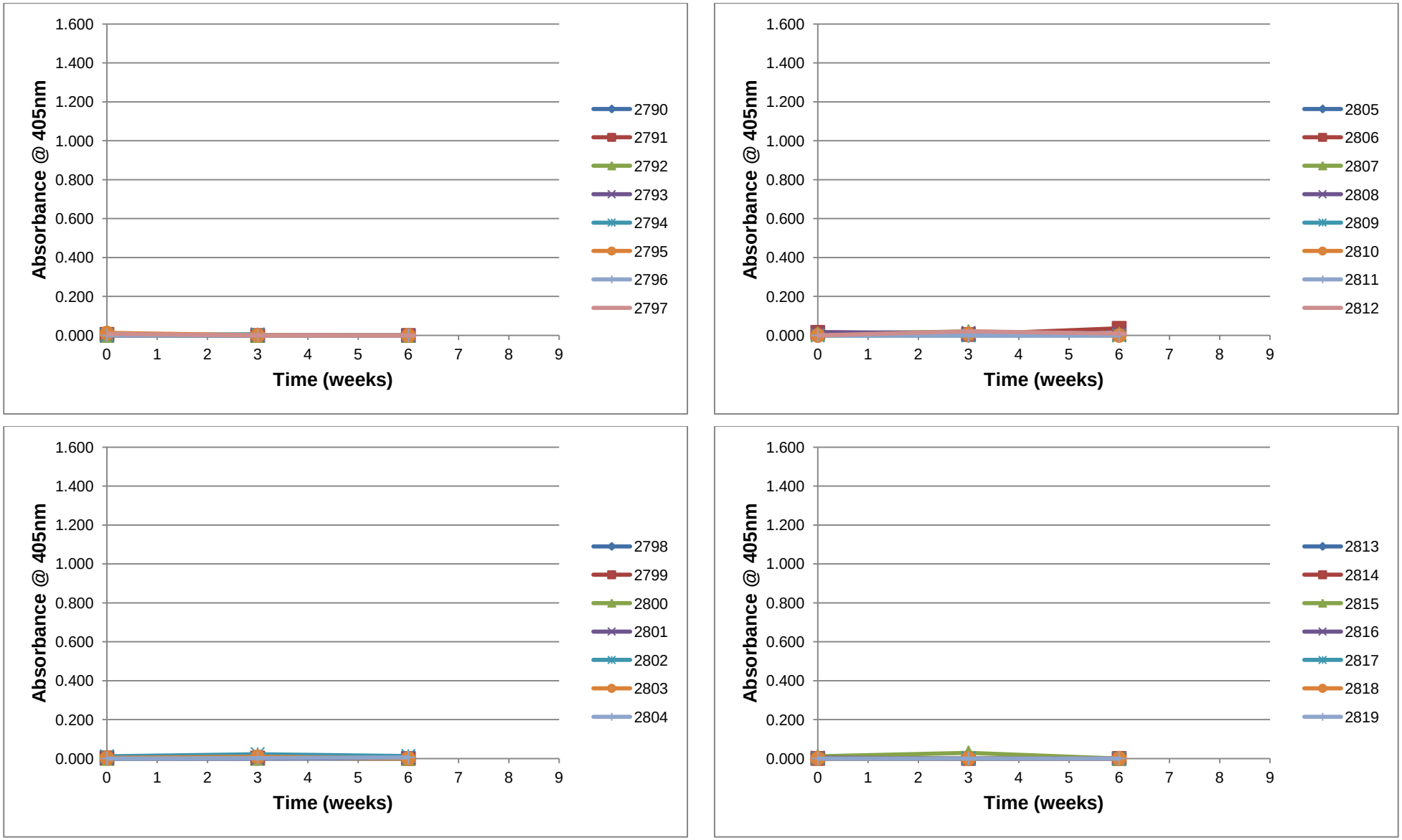

Figure B.20.: Immune responses elicited by ostriches in the control group that received no vaccinations

Mucosal immune response of ostriches against *Salmonella entrica* serovar *typhimurium* SL3261 LPS

The mucosal immune response of the ostriches against *Salmonella entrica* serovar *typhimurium* SL3261 LPS was evaluated using ELISA as described in chapter 4.2.3.5 using the swab samples collected and rabbit anti-ostrich IgA protein 2 as the biotinylated secondary antibody. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – LPS – IgA Protein 2
Oudsthoorn
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	359	0.114			
Treatment (Treatment)	3	0.005	0.002	5.66	0.0008
Time (Time)	2	0.001	0.000	0.83	0.4364
Treatment x Time	6	0.002	0.000	0.88	0.5118
Residual	348	0.107	0.000		

Grand mean = 0.009 R-squared = 0.0643 C.V. = 200.9%

LSD for Treatment = 0.0051 S.E.D = 0.0026 r = 90
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00031

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
3	0.02	163.4	1	VR1020 Plasmid
4	0.01	284.6	2	Control
1	0.01	151.4	3	Salmonella Control
2	0.01	145.6	4	Salmonella + VR1020

LSD for Time = 0.0044 S.E.D = 0.0023 r = 120
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00031

Time
Averages

Level	--- Y ---	Cv	Rank	Week
2	0.01	204.4	1	3
1	0.01	193.5	2	0
3	0.01	212.3	3	6

LSD for Treatment*Time = 0.0089 S.E.D = 0.0045 r = 30
t (2-sided a=0.050, 348 df) = 1.9668 MSE = 0.00031

Two-way table for Treatment*Time, n=30

	1	2	3
1	0.007	0.008	0.005
2	0.006	0.006	0.004
3	0.016	0.013	0.016
4	0.006	0.013	0.003

The following table the data is arranged in two columns with the ostrich identification number, Treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The Treatments consist of *Salmonella enterica* serovar *typhimurium* SL3261 control (1), mucosal VR1020 recombinant vaccine (2), intramuscular injected VR1020 recombinant vaccine (3) and the control group that received no vaccinations (4). Following the data are the graphs drawn for each ostrich depicting the immune response measured over time.

Ostrich	Trt	Time	Resp	Weight
2700	1	0	0.011	46.6
2701	1	0	0.000	45.0
2702	1	0	0.009	43.0
2703	1	0	0.000	38.0
2704	1	0	0.000	49.0
2705	1	0	0.000	50.0
2706	1	0	0.000	39.0
2707	1	0	0.000	40.0
2708	1	0	0.003	48.0
2709	1	0	0.008	43.0
2710	1	0	0.017	38.0
2711	1	0	0.000	39.0
2712	1	0	0.000	39.0
2713	1	0	0.015	36.0
2714	1	0	0.006	34.0
2715	1	0	0.011	39.0
2716	1	0	0.009	42.0
2717	1	0	0.000	46.0
2718	1	0	0.021	39.0
2719	1	0	0.036	45.0
2720	1	0	0.012	51.0
2721	1	0	0.003	40.0
2722	1	0	0.005	39.0
2723	1	0	0.002	42.0
2724	1	0	0.001	43.0
2725	1	0	0.013	43.0
2726	1	0	0.010	39.0
2727	1	0	0.006	49.0
2728	1	0	0.006	47.0
2729	1	0	0.011	39.8
2730	2	0	0.007	44.1
2731	2	0	0.011	46.0
2732	2	0	0.000	41.0
2733	2	0	0.008	45.0
2734	2	0	0.009	47.0
2735	2	0	0.007	35.0
2736	2	0	0.004	40.0
2737	2	0	0.001	41.0
2738	2	0	0.000	34.0
2739	2	0	0.000	38.0
2740	2	0	0.000	48.0
2741	2	0	0.007	40.0
2742	2	0	0.000	42.0
2743	2	0	0.010	46.0
2744	2	0	0.000	36.0
2745	2	0	0.008	49.0
2746	2	0	0.000	41.0
2747	2	0	0.013	43.0
2748	2	0	0.022	41.0
2749	2	0	0.000	36.0
2750	2	0	0.000	38.0
2751	2	0	0.000	39.0
2752	2	0	0.002	40.0
2753	2	0	0.002	42.0
2754	2	0	0.000	44.0
2755	2	0	0.042	44.0
2756	2	0	0.018	43.0
2757	2	0	0.000	38.0
2758	2	0	0.013	36.0
2759	2	0	0.003	43.0
2760	3	0	0.012	40.0
2761	3	0	0.001	40.0
2762	3	0	0.127	38.0
2763	3	0	0.000	43.0
2764	3	0	0.017	41.0
2765	3	0	0.001	37.0
2766	3	0	0.000	44.0
2767	3	0	0.003	40.0
2768	3	0	0.000	39.0
2769	3	0	0.000	39.0
2770	3	0	0.000	37.0
2771	3	0	0.000	48.0
2772	3	0	0.005	37.0
2773	3	0	0.004	37.0
2774	3	0	0.005	43.0
2775	3	0	0.000	39.0
2776	3	0	0.000	37.0
2777	3	0	0.000	39.0
2778	3	0	0.000	41.0

2779	3	0	0.045	37.0
2780	3	0	0.074	40.0
2781	3	0	0.037	39.0
2782	3	0	0.015	39.0
2783	3	0	0.017	40.0
2784	3	0	0.034	41.0
2785	3	0	0.064	41.0
2786	3	0	0.026	41.0
2787	3	0	0.005	39.0
2788	3	0	0.000	39.0
2789	3	0	0.000	39.0
2790	4	0	0.000	38.0
2791	4	0	0.000	40.0
2792	4	0	0.000	36.0
2793	4	0	0.000	38.0
2794	4	0	0.009	39.0
2795	4	0	0.000	37.0
2796	4	0	0.000	40.0
2797	4	0	0.000	37.0
2798	4	0	0.000	36.0
2799	4	0	0.000	36.0
2800	4	0	0.000	37.0
2801	4	0	0.000	37.0
2802	4	0	0.001	36.0
2803	4	0	0.000	37.0
2804	4	0	0.011	35.0
2805	4	0	0.000	43.0
2806	4	0	0.007	38.0
2807	4	0	0.016	38.0
2808	4	0	0.015	45.0
2809	4	0	0.011	37.0
2810	4	0	0.000	38.0
2811	4	0	0.000	38.0
2812	4	0	0.000	40.0
2813	4	0	0.035	38.0
2814	4	0	0.000	37.0
2815	4	0	0.007	36.0
2816	4	0	0.000	42.0
2817	4	0	0.009	37.0
2818	4	0	0.022	36.0
2819	4	0	0.008	35.0
2700	1	3	0.000	50.5
2701	1	3	0.008	45.4
2702	1	3	0.010	43.0
2703	1	3	0.000	40.6
2704	1	3	0.000	55.5
2705	1	3	0.000	46.0
2706	1	3	0.000	39.2
2707	1	3	0.000	37.8
2708	1	3	0.001	42.0
2709	1	3	0.013	44.8

2710	1	3	0.074	40.2
2711	1	3	0.000	35.2
2712	1	3	0.000	37.2
2713	1	3	0.015	33.6
2714	1	3	0.005	34.4
2715	1	3	0.009	35.0
2716	1	3	0.012	35.6
2717	1	3	0.000	39.8
2718	1	3	0.025	43.8
2719	1	3	0.012	44.6
2720	1	3	0.012	56.5
2721	1	3	0.010	39.2
2722	1	3	0.010	43.6
2723	1	3	0.003	40.2
2724	1	3	0.001	42.8
2725	1	3	0.011	45.4
2726	1	3	0.005	40.8
2727	1	3	0.005	49.6
2728	1	3	0.005	44.4
2729	1	3	0.016	41.6
2730	2	3	0.014	43.2
2731	2	3	0.011	47.4
2732	2	3	0.000	42.8
2733	2	3	0.002	46.8
2734	2	3	0.007	52.0
2735	2	3	0.021	30.4
2736	2	3	0.007	38.8
2737	2	3	0.000	46.4
2738	2	3	0.000	36.4
2739	2	3	0.000	38.2
2740	2	3	0.000	49.2
2741	2	3	0.003	43.8
2742	2	3	0.000	42.4
2743	2	3	0.006	44.8
2744	2	3	0.007	34.8
2745	2	3	0.009	50.5
2746	2	3	0.000	41.2
2747	2	3	0.009	44.4
2748	2	3	0.020	36.8
2749	2	3	0.003	39.4
2750	2	3	0.000	40.2
2751	2	3	0.003	34.8
2752	2	3	0.009	39.0
2753	2	3	0.011	41.6
2754	2	3	0.000	40.6
2755	2	3	0.043	44.4
2756	2	3	0.014	45.2
2757	2	3	0.000	40.6
2758	2	3	0.004	40.2
2759	2	3	0.000	41.0
2760	3	3	0.014	39.4

2761	3	3	0.018	42.6
2762	3	3	0.024	37.0
2763	3	3	0.025	39.8
2764	3	3	0.038	41.2
2765	3	3	0.000	37.8
2766	3	3	0.000	40.6
2767	3	3	0.002	43.2
2768	3	3	0.000	43.2
2769	3	3	0.000	38.8
2770	3	3	0.000	43.8
2771	3	3	0.002	41.4
2772	3	3	0.005	40.8
2773	3	3	0.004	37.6
2774	3	3	0.010	46.6
2775	3	3	0.000	32.0
2776	3	3	0.001	37.8
2777	3	3	0.004	41.4
2778	3	3	0.003	44.6
2779	3	3	0.030	38.0
2780	3	3	0.048	41.8
2781	3	3	0.034	39.2
2782	3	3	0.054	37.6
2783	3	3	0.008	35.0
2784	3	3	0.041	40.8
2785	3	3	0.043	42.4
2786	3	3	0.018	41.0
2787	3	3	0.004	36.2
2788	3	3	0.000	44.4
2789	3	3	0.000	40.2
2790	4	3	0.000	37.8
2791	4	3	0.000	36.8
2792	4	3	0.002	32.4
2793	4	3	0.000	34.2
2794	4	3	0.132	39.0
2795	4	3	0.000	40.2
2796	4	3	0.000	36.2
2797	4	3	0.000	34.4
2798	4	3	0.000	41.4
2799	4	3	0.000	36.4
2800	4	3	0.000	37.0
2801	4	3	0.000	33.2
2802	4	3	0.003	36.4
2803	4	3	0.000	40.6
2804	4	3	0.001	36.6
2805	4	3	0.000	41.0
2806	4	3	0.005	35.4
2807	4	3	0.019	38.2
2808	4	3	0.009	44.4
2809	4	3	0.028	35.4
2810	4	3	0.003	38.6
2811	4	3	0.003	40.2
2812	4	3	0.000	43.4
2813	4	3	0.019	37.4
2814	4	3	0.000	34.0
2815	4	3	0.017	31.0
2816	4	3	0.000	47.0
2817	4	3	0.022	39.2
2818	4	3	0.139	40.0
2819	4	3	0.000	33.0
2700	1	6	0.000	51.5
2701	1	6	0.000	49.4
2702	1	6	0.003	44.0
2703	1	6	0.000	47.6
2704	1	6	0.000	63.0
2705	1	6	0.000	49.0
2706	1	6	0.000	42.4
2707	1	6	0.001	39.6
2708	1	6	0.013	43.6
2709	1	6	0.000	49.2
2710	1	6	0.016	41.8
2711	1	6	0.001	35.8
2712	1	6	0.000	39.4
2713	1	6	0.000	32.8
2714	1	6	0.001	40.4
2715	1	6	0.010	40.8
2716	1	6	0.017	37.8
2717	1	6	0.000	44.8
2718	1	6	0.005	48.2
2719	1	6	0.014	50.0
2720	1	6	0.006	64.0
2721	1	6	0.014	41.2
2722	1	6	0.011	45.8
2723	1	6	0.000	42.0
2724	1	6	0.002	48.0
2725	1	6	0.009	51.5
2726	1	6	0.002	47.0
2727	1	6	0.022	59.0
2728	1	6	0.011	46.6
2729	1	6	0.014	43.2
2730	2	6	0.000	41.8
2731	2	6	0.006	58.0
2732	2	6	.	.
2733	2	6	0.019	51.5
2734	2	6	0.000	62.5
2735	2	6	0.006	35.4
2736	2	6	0.000	45.0
2737	2	6	0.002	50.0
2738	2	6	0.001	35.0
2739	2	6	0.000	46.6
2740	2	6	0.000	61.0
2741	2	6	0.000	50.5
2742	2	6	0.000	49.8

2743	2	6	0.003	48.6
2744	2	6	0.000	32.0
2745	2	6	0.008	52.0
2746	2	6	0.003	53.5
2747	2	6	0.000	53.5
2748	2	6	.	.
2749	2	6	0.005	46.6
2750	2	6	0.009	46.0
2751	2	6	0.007	45.0
2752	2	6	0.018	47.6
2753	2	6	0.010	52.5
2754	2	6	0.000	47.8
2755	2	6	0.014	46.0
2756	2	6	0.010	47.4
2757	2	6	0.014	45.0
2758	2	6	0.000	47.4
2759	2	6	0.000	40.8
2760	3	6	0.023	42.0
2761	3	6	0.015	44.8
2762	3	6	0.002	39.8
2763	3	6	0.031	39.2
2764	3	6	0.005	49.0
2765	3	6	0.000	38.2
2766	3	6	0.000	45.4
2767	3	6	0.000	47.8
2768	3	6	0.000	46.8
2769	3	6	0.000	39.2
2770	3	6	0.000	42.0
2771	3	6	0.003	42.8
2772	3	6	0.000	40.4
2773	3	6	0.022	33.4
2774	3	6	0.012	45.2
2775	3	6	0.000	37.8
2776	3	6	0.000	40.3
2777	3	6	0.000	44.4
2778	3	6	0.008	46.0
2779	3	6	0.040	37.0
2780	3	6	0.057	44.2
2781	3	6	0.064	45.8
2782	3	6	.	.
2783	3	6	0.013	38.0
2784	3	6	0.120	43.4
2785	3	6	0.037	48.4
2786	3	6	0.045	42.6
2787	3	6	0.001	36.6
2788	3	6	0.000	46.6
2789	3	6	0.000	47.8
2790	4	6	0.000	42.4
2791	4	6	0.000	44.4
2792	4	6	0.000	35.0
2793	4	6	0.000	33.2
2794	4	6	.	.
2795	4	6	0.000	46.6
2796	4	6	0.000	36.0
2797	4	6	0.000	35.8
2798	4	6	0.000	48.6
2799	4	6	0.000	38.6
2800	4	6	0.000	46.2
2801	4	6	0.000	38.4
2802	4	6	0.005	35.6
2803	4	6	0.000	39.4
2804	4	6	0.002	37.2
2805	4	6	0.006	48.4
2806	4	6	0.008	40.2
2807	4	6	0.009	38.4
2808	4	6	0.011	48.0
2809	4	6	0.001	38.8
2810	4	6	0.000	43.8
2811	4	6	0.000	40.2
2812	4	6	0.007	43.0
2813	4	6	0.006	38.8
2814	4	6	0.010	43.0
2815	4	6	0.000	28.2
2816	4	6	0.000	47.6
2817	4	6	0.009	36.0
2818	4	6	0.014	42.6
2819	4	6	0.000	36.4

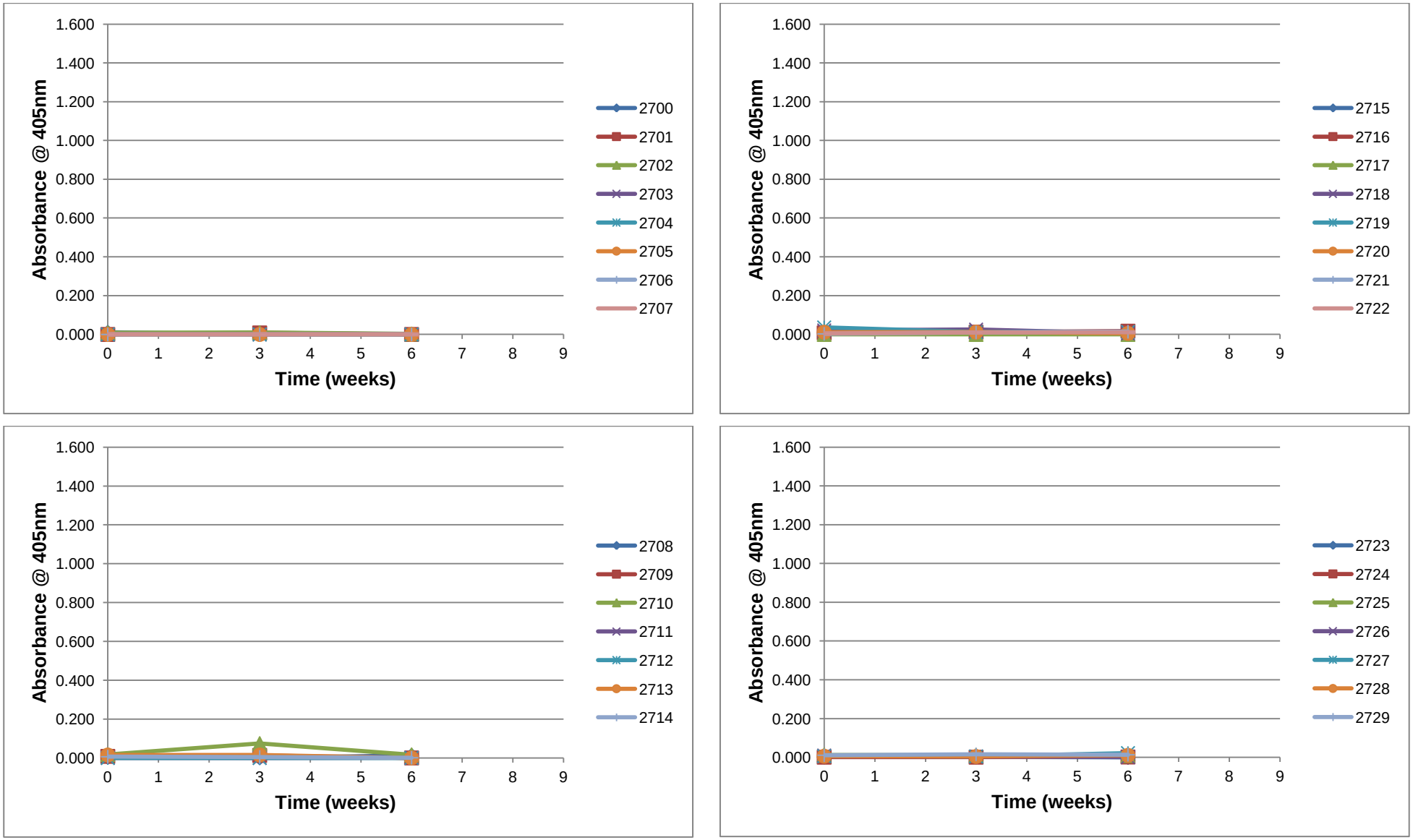

Figure B.21: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of *Salmonella enterica* serovar *typhimurium* SL3261 control

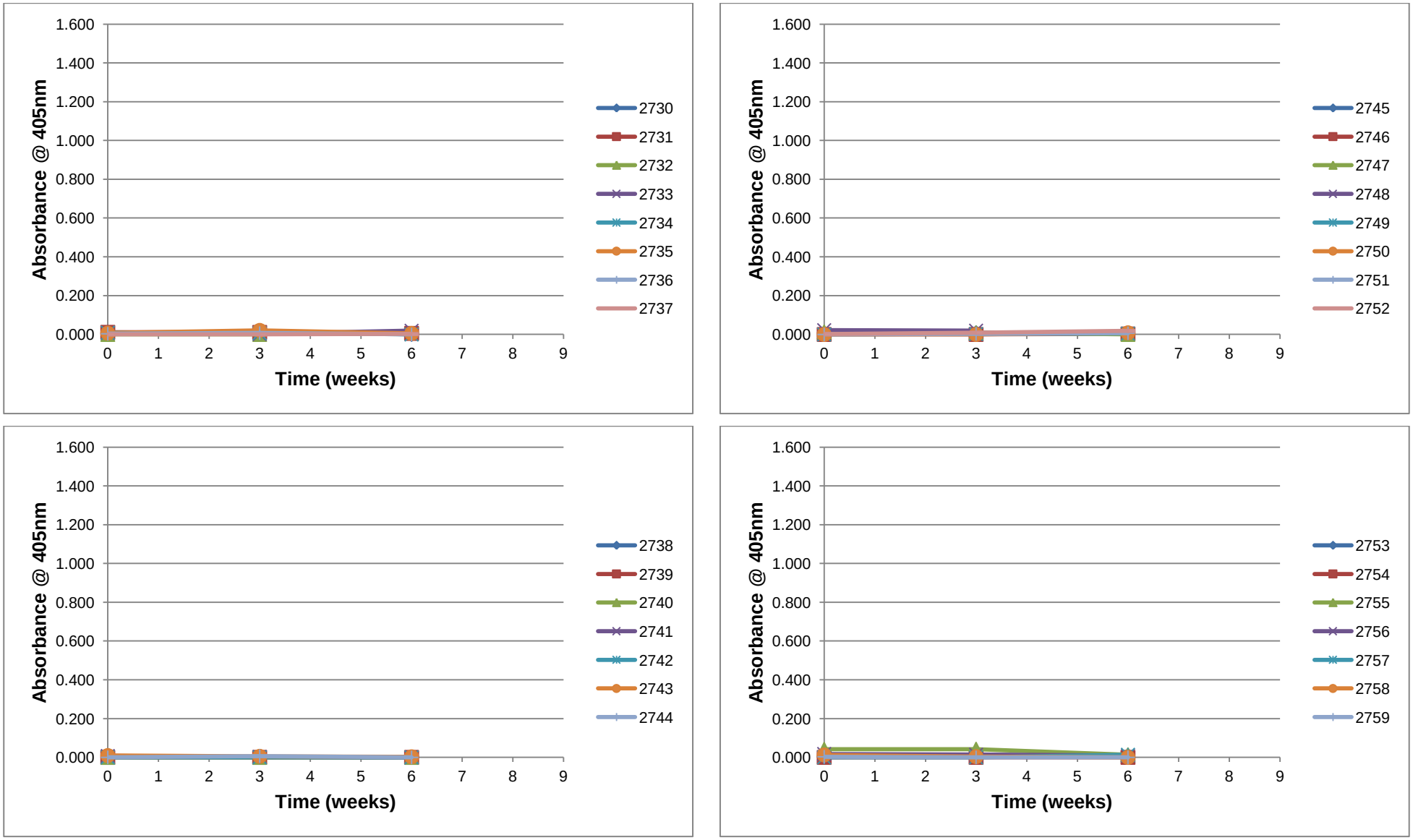

Figure B.22.: Immune responses elicited by ostriches that received 10^9 c.f.u./ml of the mucosal VR1020 recombinant vaccine

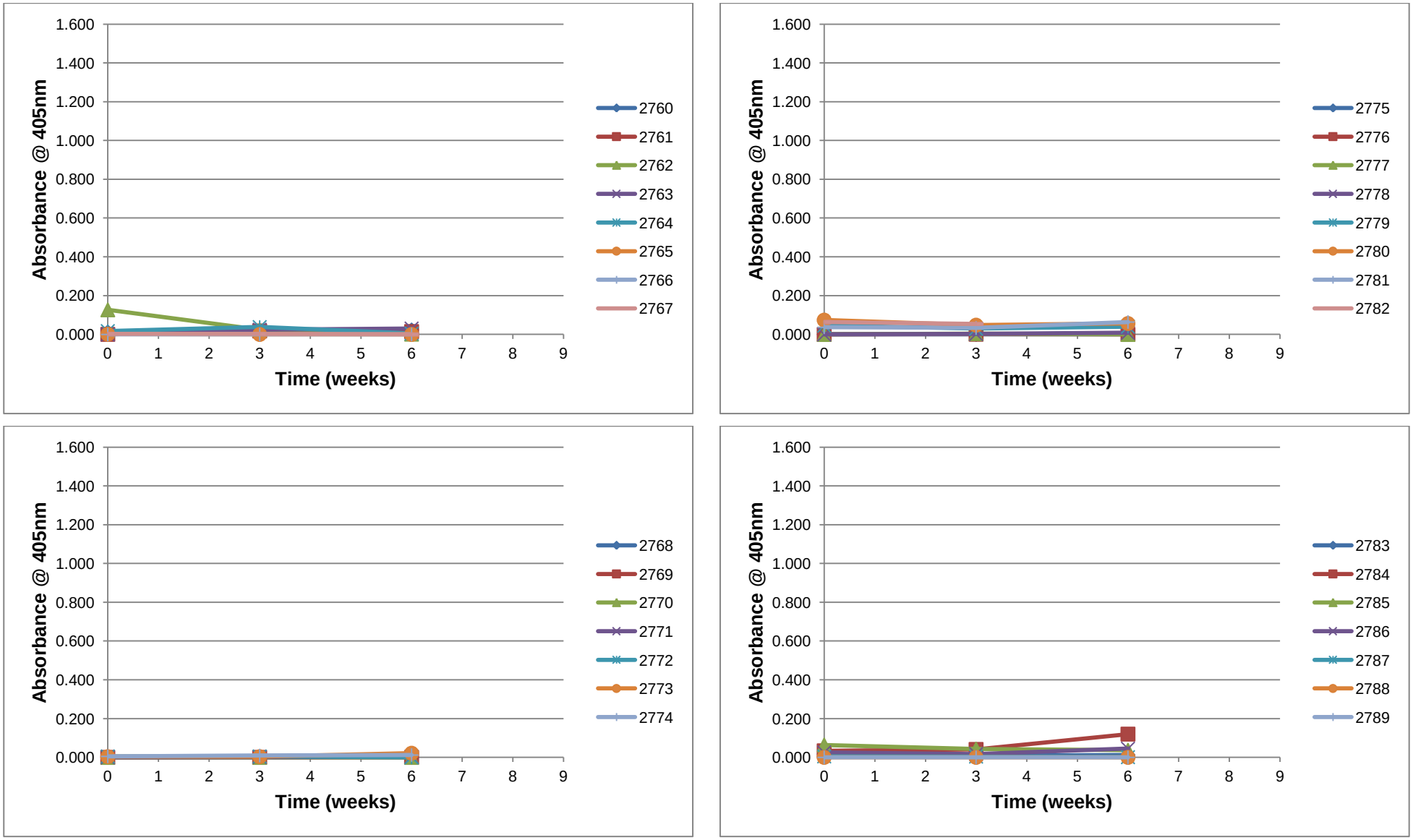

Figure B.23.: Immune responses elicited by ostriches that received 100 µg/ml of the intramuscular VR1020 recombinant vaccine

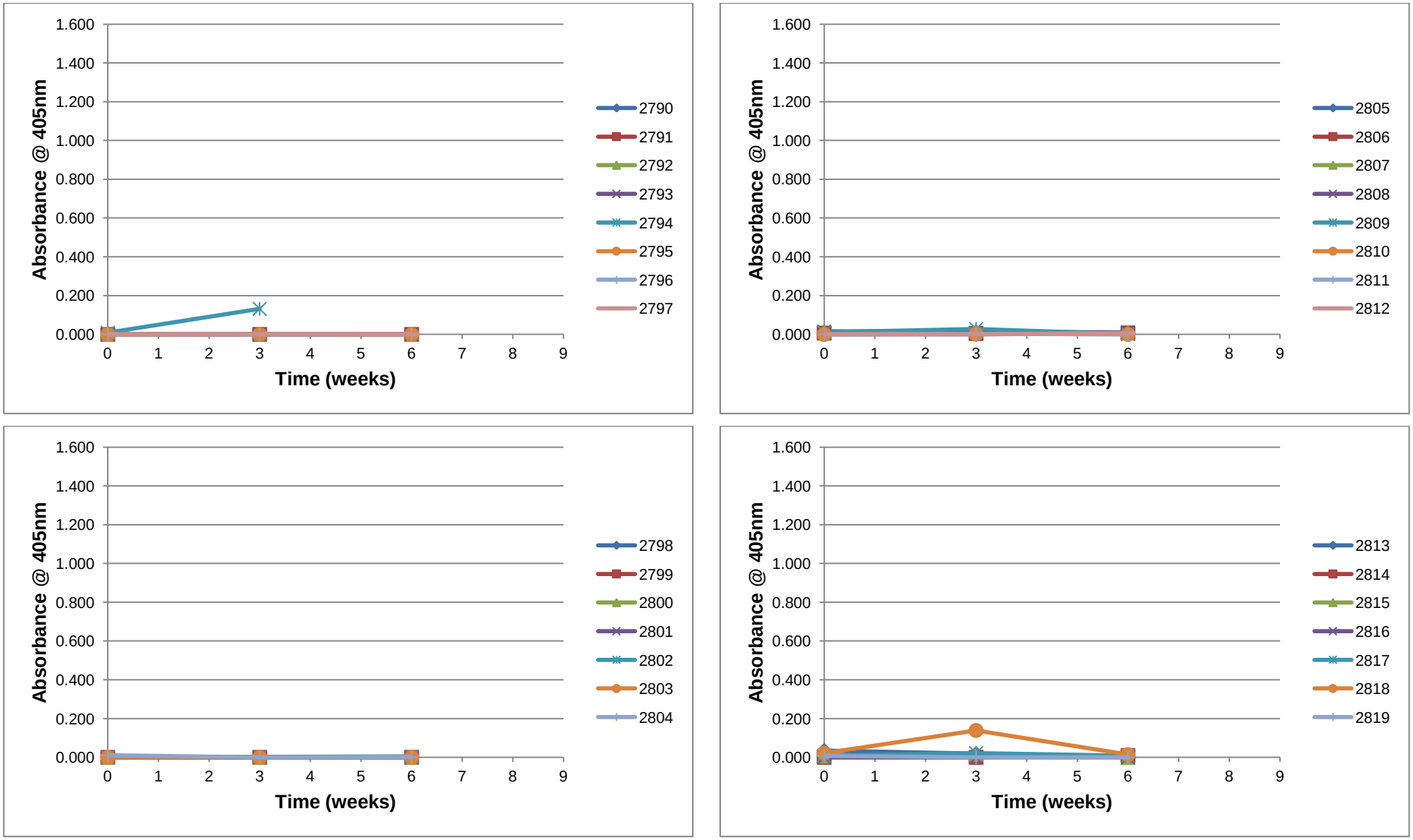

Figure B.24.: Immune responses elicited by ostriches in the control group that received no vaccinations

Addendum C: Statistical analysis of ELISA results of the dosing vaccine trial on Kromme Rhee farm in Stellenbosch, Western Cape

Humoral immune response of ostriches against *Mycoplasma struthionis*

The humoral immune response of the ostriches against the OppA protein was evaluated using ELISA as described in section 5.2.3. and the serum samples collected. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Serum - OppA
Kromme Rhee
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	329	80.023			
Treatment	10	24.964	2.496	16.70	0.0000
Time	5	7.998	1.600	10.70	0.0000
Treatment x Time	50	7.607	0.152	1.020	0.4476
Residual	264	39.454	0.149		

Grand mean = 0.708 R-squared = 0.5070 C.V. = 54.60%

LSD for Treatment = 0.1965 S.E.D = 0.0998 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.14945

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
9	1.24	31.1	1	VR1020 10^11
10	0.96	64.5	2	VR1020 10^12
8	0.91	60	3	VR1020 10^10
6	0.88	43	4	Control
11	0.77	76	5	VR1020 10^13
7	0.74	62.6	6	VR1020 10^9
5	0.73	50.8	7	VR1012 10^13
3	0.47	60.8	8	VR1012 10^11
4	0.41	58.2	9	VR1012 10^12
1	0.36	54.2	10	VR1012 10^9
2	0.31	76.2	11	VR1012 10^10

LSD for Time = 0.1452 S.E.D = 0.0737 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.14945

Time
Averages

Level	--- Y ---	Cv	Rank	Week
1	0.88	54.1	1	0
2	0.86	61.6	2	1
4	0.78	67.8	3	3
3	0.74	65.1	4	2
5	0.51	79.7	5	6
6	0.49	80.9	6	9

LSD for Treatment*Time = 0.4814 S.E.D = 0.2445 r = 5
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.14945

Two-way table for Treatment*Time, n=5

	1	2	3	4	5	6
1	0.462	0.292	0.334	0.462	0.264	0.368
2	0.446	0.224	0.284	0.386	0.248	0.3
3	0.472	0.476	0.436	0.438	0.582	0.426
4	0.438	0.402	0.38	0.548	0.378	0.342
5	0.878	1.01	0.692	0.706	0.624	0.496
6	0.92	1.138	1.136	1.064	0.54	0.5
7	0.966	1.074	0.892	0.73	0.392	0.38
8	1.222	1.158	1.1	1.092	0.354	0.51
9	1.326	1.476	1.152	1.372	1.09	1.01
10	1.078	1.226	1.116	1.11	0.732	0.472
11	1.422	0.954	0.67	0.632	0.37	0.56
	1	2	3	4	5	6

A N A L Y S I S O F V A R I A N C E
Variable: WEIGHT

Source	df	SS	MS	F-value	Pr>F
Total	329	194151.1			
Treatment	10	1971.853	197.185	1.75	0.0705
Time	5	157582.3	31546.45	279.33	0.0000
Treatment x Time	50	4810.577	96.212	0.85	0.7473
Residual	264	29786.42	112.827		

Grand mean = 21.664 R-squared = 0.8466 C.V. = 49.03%

LSD for Treatment = 5.4001 S.E.D = 2.7426 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 112.82733

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
6	25.24	103.2	1	Control
9	24.26	102.9	2	VR1020 10^11
8	26.93	110.4	3	VR1020 10^10
4	22.93	104	4	VR1020 10^12
5	22.9	109.6	5	VR1020 10^13
1	21.79	110	6	VR1020 10^9
3	21.07	109.7	7	VR1012 10^11
10	20.53	118.5	8	VR1012 10^12
7	20.32	126.5	9	VR1012 10^9
2	18.8	126.3	10	VR1012 10^10
11	16.53	135.8	11	VR1012 10^13

LSD for Time = 3.9883 S.E.D = 2.0255 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 112.82733

Time
Averages

Level	--- Y ---	Cv	Rank	Time
1	48.98	6.2	1	0
3	41.26	31.2	2	2
6	39.74	56.4	3	9
2	0.00	-9.0	4	1
4	0.00	-9.0	5	3
5	0.00	-9.0	6	6

LSD for Treatment*Time = 13.2276 S.E.D = 6.718 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 112.82733

Two-way table for Treatment*Time, n=30

	1	2	3	4	5	6
1	47.120	0.000	43.340	0.000	0.000	40.280
2	47.280	0.000	34.520	0.000	0.000	31.000
3	47.600	0.000	40.880	0.000	0.000	37.960
4	47.640	0.000	44.060	0.000	0.000	45.900
5	49.120	0.000	45.760	0.000	0.000	42.540
6	53.440	0.000	47.820	0.000	0.000	50.160
7	49.960	0.000	47.360	0.000	0.000	24.600
8	50.400	0.000	46.560	0.000	0.000	46.600
9	50.040	0.000	45.700	0.000	0.000	49.800
10	48.440	0.000	34.240	0.000	0.000	40.520
11	47.760	0.000	23.640	0.000	0.000	27.800

The following table contains the input data for the ANOVA, and is arranged in two columns with the ostrich identification number, treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The treatments i.e. vaccine doses, consisted of VR1012 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (1), $1x10^{10}$ c.f.u./ml (2), $1x10^{11}$ c.f.u./ml (3), $1x10^{12}$ c.f.u./ml (4) and $1x10^{13}$ c.f.u./ml (5); *Salmonella enterica* serovar *typhimurium* SL3261 control (6), VR1020 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (7), $1x10^{10}$ c.f.u./ml (8), $1x10^{11}$ c.f.u./ml (9), $1x10^{12}$ c.f.u./ml (10) and $1x10^{13}$ c.f.u./ml (11). Following the data arethe graphs drawn for each ostrich depicting the absorbance measured over time.

Ostrich	Trt	Time	Resp	Weight
2	1	0	0.560	44.6
10	1	0	0.397	47.6
12	1	0	0.346	50.4
18	1	0	0.597	45.2
19	1	0	0.402	47.8
7	2	0	0.535	44.2
9	2	0	0.028	46.8
11	2	0	0.575	48.4
13	2	0	0.402	50
14	2	0	0.682	47
48	3	0	0.153	48.6
56	3	0	1.062	48
57	3	0	0.439	47.6
58	3	0	0.526	47.6
59	3	0	0.184	46.2
44	4	0	0.421	48.2
45	4	0	0.676	46.6
51	4	0	0.258	45.6
52	4	0	0.464	46.8
54	4	0	0.369	51
23	5	0	1.107	51.6
32	5	0	1.468	46.6
35	5	0	0.614	50.4
37	5	0	0.481	49.8
40	5	0	0.721	47.2
3	6	0	1.028	52.4
20	6	0	1.141	52.4
41	6	0	0.760	51.8
47	6	0	0.510	54.8
55	6	0	1.162	55.8
53	6	0	0.534	55.4
21	7	0	1.081	48.8
24	7	0	0.667	50
26	7	0	1.439	52.8
31	7	0	0.803	47
36	7	0	0.838	51.2
25	8	0	2.159	48.2
29	8	0	0.758	46.8
30	8	0	1.081	50
33	8	0	1.602	53.8
89	8	0	0.511	53.2
81	9	0	1.778	44
82	9	0	1.688	50.8
64	9	0	0.863	54
88	9	0	1.354	50.6
91	9	0	0.954	50.8
74	10	0	1.075	55.6
66	10	0	0.658	48.2
71	10	0	1.268	44.8
77	10	0	1.483	49
79	10	0	0.904	44.6
65	11	0	1.397	50.4
69	11	0	1.402	50.6
70	11	0	1.244	47.6
76	11	0	1.533	44
78	11	0	1.544	46.2
2	1	1	0.213	.
10	1	1	0.263	.
12	1	1	0.349	.
18	1	1	0.344	.
19	1	1	0.295	.
7	2	1	0.213	.
9	2	1	.	.
11	2	1	0.263	.
13	2	1	0.295	.
14	2	1	0.349	.
48	3	1	0.277	.
56	3	1	0.772	.
57	3	1	0.585	.
58	3	1	0.485	.
59	3	1	0.252	.
44	4	1	0.344	.
45	4	1	0.783	.
51	4	1	0.195	.
52	4	1	0.342	.
54	4	1	0.347	.
23	5	1	0.898	.
32	5	1	1.596	.
35	5	1	1.074	.
37	5	1	0.635	.
40	5	1	0.836	.
3	6	1	1.039	.
20	6	1	1.292	.
41	6	1	0.598	.
47	6	1	1.482	.
55	6	1	1.280	.
53	6	1	0.896	.
21	7	1	1.278	.
24	7	1	0.889	.
26	7	1	1.133	.
31	7	1	1.256	.
36	7	1	0.805	.
25	8	1	1.544	.
29	8	1	0.858	.
30	8	1	0.607	.
33	8	1	1.797	.
89	8	1	0.980	.
81	9	1	1.141	.
82	9	1	1.733	.
64	9	1	1.152	.
88	9	1	1.601	.
91	9	1	1.760	.
74	10	1	0.851	.
66	10	1	0.898	.
71	10	1	1.182	.
77	10	1	2.249	.
79	10	1	0.950	.
65	11	1	.	.
69	11	1	1.232	.
70	11	1	0.714	.
76	11	1	1.301	.
78	11	1	1.527	.
2	1	2	0.370	41.8
10	1	2	0.244	51.5
12	1	2	0.555	47
18	1	2	0.288	40.2
19	1	2	0.206	36.2
7	2	2	0.370	43.2
9	2	2	.	.
11	2	2	0.244	44.4
13	2	2	0.254	44.4
14	2	2	0.555	40.6
48	3	2	0.220	47
56	3	2	0.683	40.6
57	3	2	0.546	44.2

58	3	2	0.480	36
59	3	2	0.247	36.6
44	4	2	0.288	40
45	4	2	0.649	39.8
51	4	2	0.307	40
52	4	2	0.375	58.5
54	4	2	0.272	42
23	5	2	0.762	47.6
32	5	2	1.027	45
35	5	2	0.665	47.8
37	5	2	0.451	47
40	5	2	0.552	41.4
3	6	2	0.874	53
20	6	2	1.357	47.4
41	6	2	0.871	41.2
47	6	2	1.532	47
55	6	2	1.048	50.5
53	6	2	1.153	47.2
21	7	2	0.847	51
24	7	2	1.277	55.5
26	7	2	1.294	42.8
31	7	2	0.489	35
36	7	2	0.550	52.5
25	8	2	1.399	34.6
29	8	2	1.097	45
30	8	2	0.814	48.2
33	8	2	1.624	51
89	8	2	0.566	54
81	9	2	1.349	46.8
82	9	2	1.314	46.2
64	9	2	0.796	45.8
88	9	2	1.147	52.5
91	9	2	1.150	37.2
74	10	2	1.443	49
66	10	2	0.757	42.6
71	10	2	.	.
77	10	2	2.117	39.2
79	10	2	1.263	40.4
65	11	2	.	.
69	11	2	1.129	40.6
70	11	2	1.179	41.4
76	11	2	1.043	36.2
78	11	2	.	.
2	1	3	0.657	.
10	1	3	0.290	.
12	1	3	0.868	.
18	1	3	0.305	.
19	1	3	0.178	.
7	2	3	0.633	.
9	2	3	.	.
11	2	3	0.314	.
13	2	3	0.220	.
14	2	3	0.774	.
48	3	3	0.204	.
56	3	3	0.400	.
57	3	3	0.937	.
58	3	3	0.425	.
59	3	3	0.220	.
44	4	3	0.160	.
45	4	3	0.793	.
51	4	3	1.040	.
52	4	3	0.554	.
54	4	3	0.203	.

23	5	3	0.752	.
32	5	3	0.983	.
35	5	3	0.463	.
37	5	3	0.466	.
40	5	3	0.869	.
3	6	3	1.343	.
20	6	3	1.309	.
41	6	3	0.454	.
47	6	3	1.016	.
55	6	3	1.204	.
53	6	3	0.989	.
21	7	3	0.840	.
24	7	3	1.118	.
26	7	3	1.200	.
31	7	3	.	.
36	7	3	0.492	.
25	8	3	1.657	.
29	8	3	1.041	.
30	8	3	0.817	.
33	8	3	1.405	.
89	8	3	0.529	.
81	9	3	0.966	.
82	9	3	1.097	.
64	9	3	1.257	.
88	9	3	1.682	.
91	9	3	1.846	.
74	10	3	1.574	.
66	10	3	0.626	.
71	10	3	.	.
77	10	3	2.323	.
79	10	3	1.029	.
65	11	3	.	.
69	11	3	1.410	.
70	11	3	0.749	.
76	11	3	1.002	.
78	11	3	.	.
2	1	6	0.354	.
10	1	6	0.301	.
12	1	6	0.473	.
18	1	6	0.204	.
19	1	6	.	.
7	2	6	0.354	.
9	2	6	.	.
11	2	6	0.301	.
13	2	6	0.118	.
14	2	6	0.473	.
48	3	6	0.185	.
56	3	6	0.628	.
57	3	6	1.139	.
58	3	6	0.658	.
59	3	6	0.287	.
44	4	6	0.204	.
45	4	6	0.847	.
51	4	6	0.187	.
52	4	6	0.499	.
54	4	6	0.145	.
23	5	6	.	.
32	5	6	0.689	.
35	5	6	1.073	.
37	5	6	0.655	.
40	5	6	0.700	.
3	6	6	0.688	.
20	6	6	0.284	.

41	6	6	0.317	.
47	6	6	0.680	.
55	6	6	0.729	.
53	6	6	0.705	.
21	7	6	0.215	.
24	7	6	0.794	.
26	7	6	.	.
31	7	6	.	.
36	7	6	0.945	.
25	8	6	.	.
29	8	6	0.516	.
30	8	6	0.294	.
33	8	6	0.560	.
89	8	6	0.396	.
81	9	6	1.243	.
82	9	6	0.481	.
64	9	6	0.579	.
88	9	6	1.517	.
91	9	6	1.626	.
74	10	6	0.639	.
66	10	6	0.592	.
71	10	6	.	.
77	10	6	1.404	.
79	10	6	1.029	.
65	11	6	.	.
69	11	6	0.853	.
70	11	6	0.454	.
76	11	6	0.548	.
78	11	6	.	.
2	1	9	0.524	48
10	1	9	0.205	59
12	1	9	0.774	47.8
18	1	9	0.337	46.6
19	1	9	.	.
7	2	9	0.524	39
9	2	9	.	.
11	2	9	0.205	63
13	2	9	.	.
14	2	9	0.774	53
48	3	9	0.214	53
56	3	9	0.337	44.4
57	3	9	0.805	44.4
58	3	9	0.766	48
59	3	9	.	.
44	4	9	0.337	39.2
45	4	9	0.522	51.5
51	4	9	0.100	39.4
52	4	9	0.649	61
54	4	9	0.098	38.4
23	5	9	.	.
32	5	9	1.259	49.8
35	5	9	0.666	53
37	5	9	0.383	48.4
40	5	9	0.173	61.5
3	6	9	0.795	44.8
20	6	9	0.332	38.6
41	6	9	0.335	55
47	6	9	0.653	64
55	6	9	0.381	48.4
53	6	9	.	.
21	7	9	.	.
24	7	9	0.854	63
26	7	9	.	.
31	7	9	.	.
36	7	9	1.054	60
25	8	9	.	.
29	8	9	0.329	59
30	8	9	0.789	54
33	8	9	0.991	60.5
89	8	9	0.438	59.5
81	9	9	1.313	43.4
82	9	9	1.271	57.5
64	9	9	0.641	42.6
88	9	9	0.594	53
91	9	9	1.239	52.5
74	10	9	0.582	57.5
66	10	9	0.376	62.5
71	10	9	.	.
77	10	9	0.801	39
79	10	9	0.602	43.6
65	11	9	.	.
69	11	9	0.820	52
70	11	9	1.313	54
76	11	9	0.672	33
78	11	9	.	.

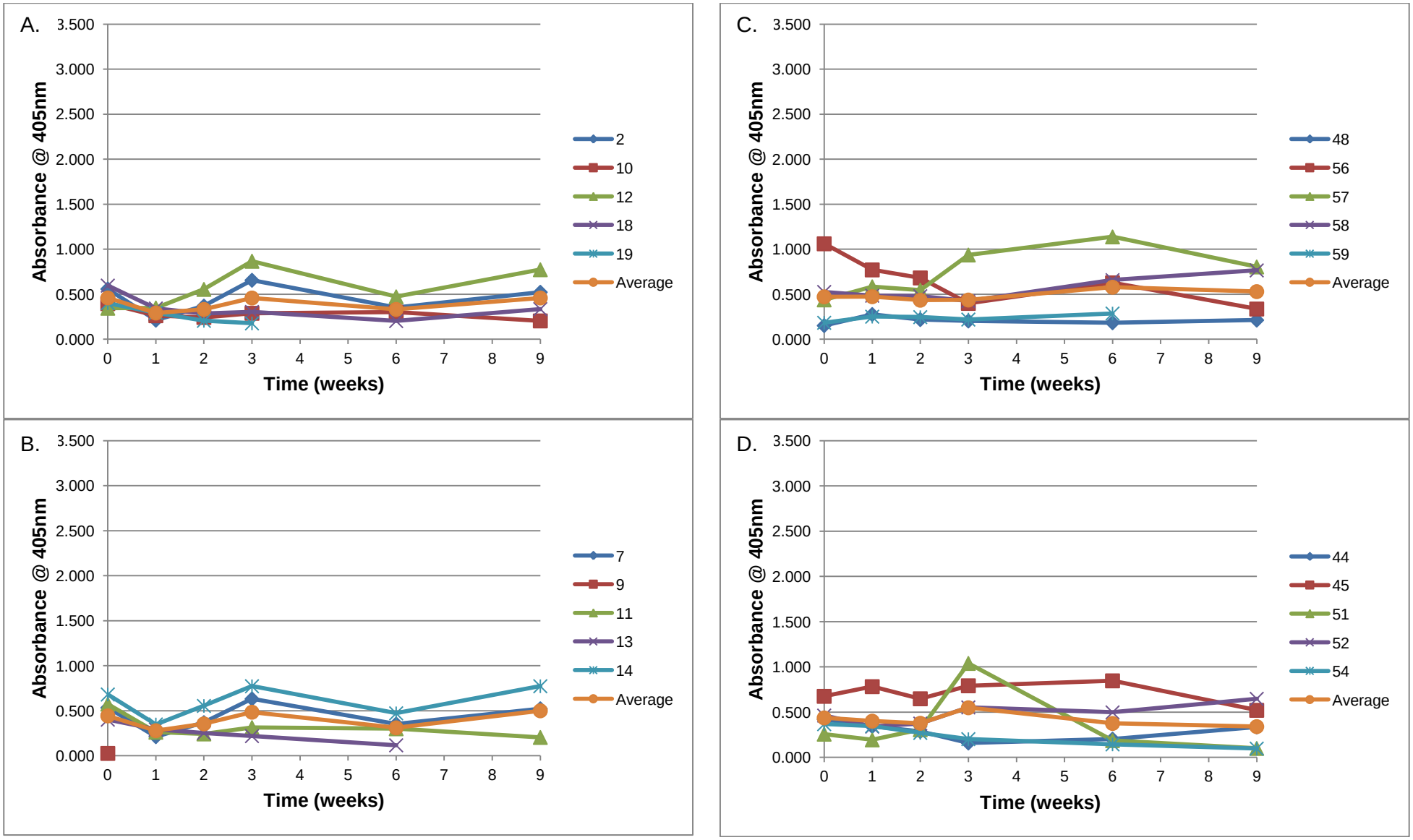

Figure C.1: Humoral immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^9 c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. 10^{10} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, C. 10^{11} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, D. Received a 10^{12} c.f.u./ml dose of the VR1012 mucosal DNA vaccine.

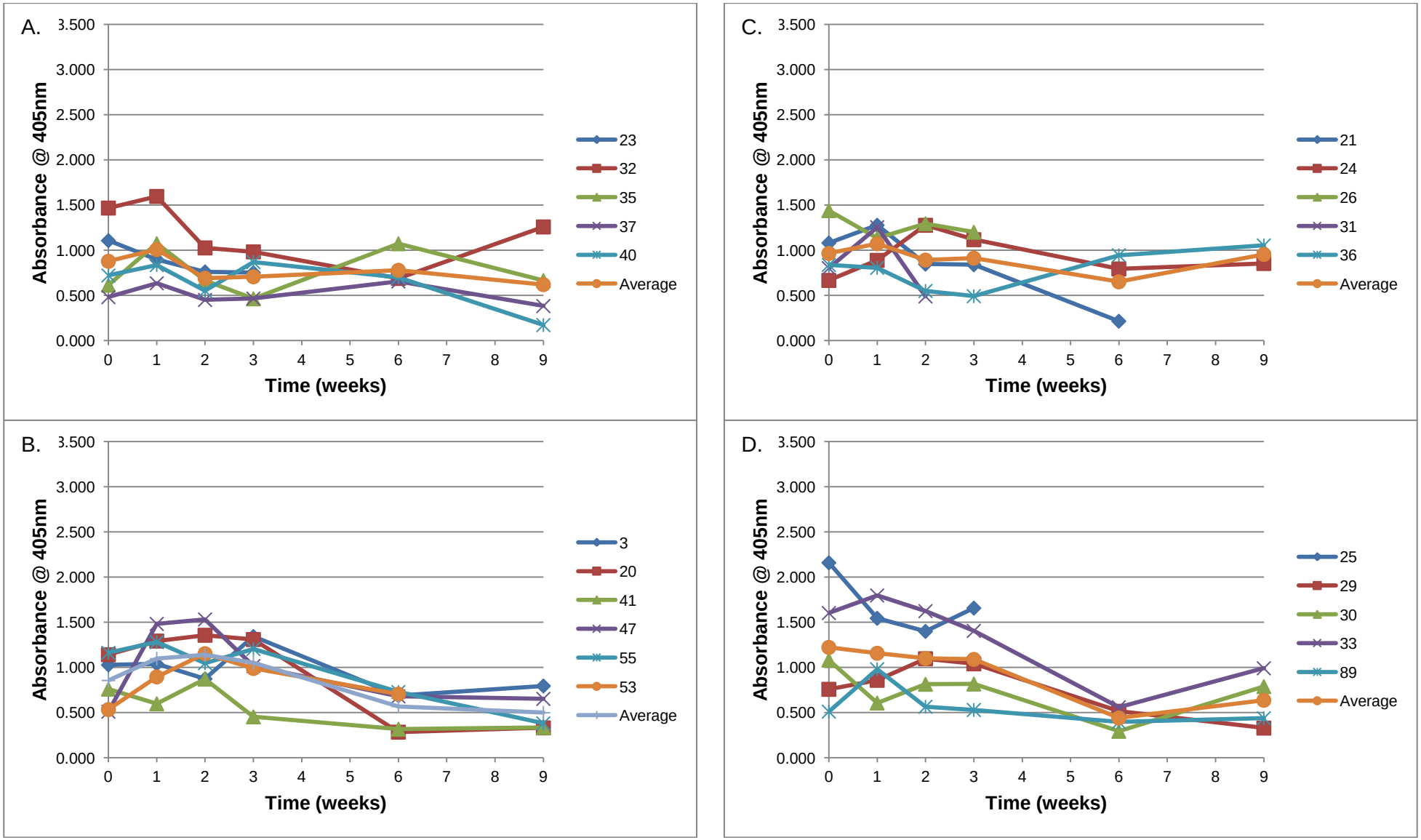

Figure C.2: Humoral immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{13} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. Control group, C. 10^{9} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, D. Received a 10^{10} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

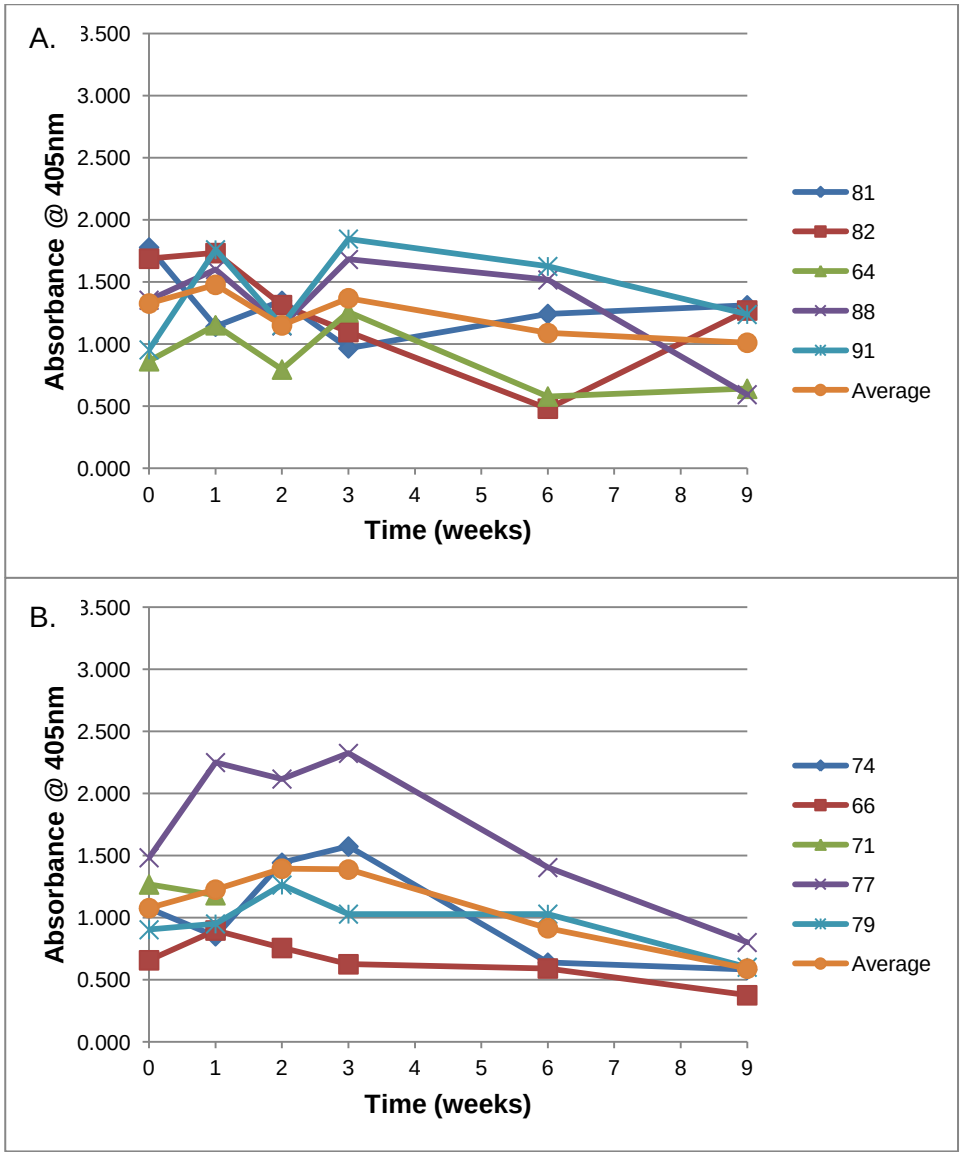

Figure C.3: Humoral immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{11} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, B. 10^{12} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, C. 10^{13} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

Mucosal immune response of ostriches against *Mycoplasma struthionis*

The mucosal immune response of ostriches against the OppA protein was evaluated using ELISA as described in section 5.2.3, using the secondary antibody rabbit anti-ostrich IgA protein 1. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – OppA – IgA Protein 1
Kromme Rhee
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	329	0.021			
Treatment	10	0.002	0.000	2.83	0.0023
Time	5	0.000	0.000	0.37	0.8674
Treatment x Time	50	0.003	0.000	0.91	0.6448
Residual	264	0.016	0.000		

Grand mean = 0.003 R-squared = 0.2230 C.V. = 266.50%

LSD for Treatment = 0.004 S.E.D = 0.0020 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00006

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
6	0.01	243.5	1	Control
9	0.01	173.4	2	VR1020 10^11
7	0.01	212.6	3	VR1020 10^9
4	0.00	124.6	4	VR1012 10^12
3	0.00	178.3	5	VR1012 10^11
10	0.00	243.6	6	VR1020 10^12
5	0.00	242.1	7	VR1012 10^13
11	0.00	325.6	8	VR1020 10^13
8	0.00	380.6	9	VR1020 10^10
2	0.00	547.7	10	VR1012 10^10
1	0.00	-9.0	11	VR1012 10^9

LSD for Time = 0.0029 S.E.D = 0.0015 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00006

Time
Averages

Level	--- Y ---	Cv	Rank	Week
4	0	312.7	1	3
5	0	226.8	2	6
3	0	249	3	2
6	0	205.2	4	9
1	0	274.9	5	0
2	0	314.2	6	1

LSD for Treatment*Time = 0.0098 S.E.D = 0.0050 r = 5

t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00006

Two-way table for Treatment*Time, n=5

	1	2	3	4	5	6
1	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.002	0.000	0.000
3	0.000	0.000	0.004	0.006	0.004	0.004
4	0.004	0.002	0.006	0.004	0.004	0.004
5	0.000	0.000	0.006	0.004	0.002	0.000
6	0.004	0.004	0.000	0.016	0.010	0.004
7	0.012	0.014	0.008	0.000	0.000	0.002
8	0.000	0.000	0.000	0.000	0.002	0.002
9	0.002	0.004	0.002	0.008	0.014	0.008
10	0.002	0.000	0.002	0.000	0.004	0.006
11	0.004	0.000	0.002	0.002	0.000	0.000

The following table contains the input data for the ANOVA and is arranged in two columns with the ostrich identification number, treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The treatments consist of VR1012 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (1), $1x10^{10}$ c.f.u./ml (2), $1x10^{11}$ c.f.u./ml (3), $1x10^{12}$ c.f.u./ml (4) and $1x10^{13}$ c.f.u./ml (5); *Salmonella enterica* serovar *typhimurium* SL3261 control (6), VR1020 naked DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (7), $1x10^{10}$ c.f.u./ml (8), $1x10^{11}$ c.f.u./ml (9), $1x10^{12}$ c.f.u./ml (10) and $1x10^{13}$ c.f.u./ml (11). Following the data are the graphs drawn for each ostrich depicting the absorbance measured over time.

Ostrich	Trt	Time	Resp	Weight
2	1	0	0.000	44.6
10	1	0	0.000	47.6
12	1	0	0.000	50.4
18	1	0	0.000	45.2
19	1	0	0.000	47.8
7	2	0	0.002	44.2
9	2	0	0.000	46.8
11	2	0	0.000	48.4
13	2	0	0.000	50
14	2	0	0.000	47
48	3	0	0.000	48.6
56	3	0	0.000	48
57	3	0	0.000	47.6
58	3	0	0.004	47.6
59	3	0	0.004	46.2
44	4	0	0.006	48.2
45	4	0	0.003	46.6
51	4	0	0.009	45.6
52	4	0	0.000	46.8
54	4	0	0.003	51
23	5	0	0.002	51.6
32	5	0	0.000	46.6
35	5	0	0.000	50.4
37	5	0	0.000	49.8
40	5	0	0.000	47.2
3	6	0	0.000	52.4
20	6	0	0.000	52.4
41	6	0	0.008	51.8
47	6	0	0.000	54.8
55	6	0	0.009	55.8
53	6	0	0.000	55.4
21	7	0	0.000	48.8
24	7	0	0.000	50
26	7	0	0.000	52.8
31	7	0	0.043	47
36	7	0	0.018	51.2
25	8	0	0.000	48.2
29	8	0	0.000	46.8
30	8	0	0.000	50
33	8	0	0.000	53.8
89	8	0	0.000	53.2
81	9	0	0.002	44
82	9	0	0.001	50.8
64	9	0	0.000	54
88	9	0	0.006	50.6
91	9	0	0.000	50.8
74	10	0	0.000	55.6
66	10	0	0.000	48.2
71	10	0	0.000	44.8
77	10	0	0.000	49
79	10	0	0.009	44.6
65	11	0	0.000	50.4
69	11	0	0.020	50.6
70	11	0	0.000	47.6
76	11	0	0.000	44
78	11	0	0.000	46.2
2	1	1	0.000	.
10	1	1	0.000	.
12	1	1	0.000	.
18	1	1	0.000	.
19	1	1	0.000	.
7	2	1	0.000	.
9	2	1	.	.
11	2	1	0.000	.
13	2	1	0.000	.
14	2	1	0.000	.
48	3	1	0.000	.
56	3	1	0.000	.
57	3	1	0.000	.
58	3	1	0.003	.
59	3	1	0.003	.
44	4	1	0.008	.
45	4	1	0.004	.
51	4	1	0.000	.
52	4	1	0.000	.
54	4	1	0.000	.
23	5	1	0.004	.
32	5	1	0.000	.
35	5	1	0.000	.
37	5	1	0.000	.
40	5	1	0.000	.
3	6	1	0.000	.
20	6	1	0.000	.
41	6	1	0.022	.
47	6	1	0.000	.
55	6	1	0.000	.
53	6	1	0.000	.
21	7	1	0.000	.
24	7	1	0.000	.
26	7	1	0.008	.
31	7	1	0.021	.
36	7	1	0.043	.
25	8	1	0.000	.
29	8	1	0.000	.
30	8	1	0.000	.
33	8	1	0.000	.
89	8	1	0.004	.
81	9	1	0.008	.
82	9	1	0.001	.
64	9	1	0.000	.
88	9	1	0.011	.
91	9	1	0.000	.
74	10	1	0.000	.
66	10	1	0.000	.
71	10	1	0.000	.
77	10	1	0.001	.
79	10	1	0.000	.
65	11	1	.	.
69	11	1	0.000	.
70	11	1	0.000	.
76	11	1	0.000	.
78	11	1	0.000	.
2	1	2	0.000	41.8
10	1	2	0.000	51.5
12	1	2	0.000	47
18	1	2	0.000	40.2
19	1	2	0.000	36.2
7	2	2	0.000	43.2
9	2	2	.	.
11	2	2	0.000	44.4
13	2	2	0.000	44.4
14	2	2	0.000	40.6
48	3	2	0.000	47
56	3	2	0.000	40.6
57	3	2	0.000	44.2

58	3	2	0.009	36
59	3	2	0.005	36.6
44	4	2	0.008	40
45	4	2	0.010	39.8
51	4	2	0.012	40
52	4	2	0.000	58.5
54	4	2	0.000	42
23	5	2	0.009	47.6
32	5	2	0.000	45
35	5	2	0.000	47.8
37	5	2	0.017	47
40	5	2	0.000	41.4
3	6	2	0.000	53
20	6	2	0.000	47.4
41	6	2	0.000	41.2
47	6	2	0.000	47
55	6	2	0.004	50.5
53	6	2	0.000	47.2
21	7	2	0.000	51
24	7	2	0.000	55.5
26	7	2	0.003	42.8
31	7	2	0.043	35
36	7	2	0.003	52.5
25	8	2	0.000	34.6
29	8	2	0.000	45
30	8	2	0.000	48.2
33	8	2	0.000	51
89	8	2	0.000	54
81	9	2	0.000	46.8
82	9	2	0.005	46.2
64	9	2	0.000	45.8
88	9	2	0.000	52.5
91	9	2	0.000	37.2
74	10	2	0.000	49
66	10	2	0.000	42.6
71	10	2	.	.
77	10	2	0.006	39.2
79	10	2	0.000	40.4
65	11	2	.	.
69	11	2	0.009	40.6
70	11	2	0.000	41.4
76	11	2	0.000	36.2
78	11	2	.	.
2	1	3	0.000	.
10	1	3	0.000	.
12	1	3	0.000	.
18	1	3	0.000	.
19	1	3	0.000	.
7	2	3	0.000	.
9	2	3	.	.
11	2	3	0.000	.
13	2	3	0.005	.
14	2	3	0.000	.
48	3	3	0.000	.
56	3	3	0.002	.
57	3	3	0.000	.
58	3	3	0.018	.
59	3	3	0.007	.
44	4	3	0.000	.
45	4	3	0.010	.
51	4	3	0.010	.
52	4	3	0.000	.
54	4	3	0.000	.

23	5	3	0.000	.
32	5	3	0.006	.
35	5	3	0.000	.
37	5	3	0.009	.
40	5	3	0.000	.
3	6	3	0.000	.
20	6	3	0.000	.
41	6	3	0.079	.
47	6	3	0.000	.
55	6	3	0.000	.
53	6	3	0.000	.
21	7	3	0.000	.
24	7	3	0.000	.
26	7	3	0.000	.
31	7	3	.	.
36	7	3	0.000	.
25	8	3	0.000	.
29	8	3	0.000	.
30	8	3	0.000	.
33	8	3	0.000	.
89	8	3	0.000	.
81	9	3	0.000	.
82	9	3	0.003	.
64	9	3	0.008	.
88	9	3	0.029	.
91	9	3	0.000	.
74	10	3	0.000	.
66	10	3	0.000	.
71	10	3	.	.
77	10	3	0.000	.
79	10	3	0.000	.
65	11	3	.	.
69	11	3	0.007	.
70	11	3	0.000	.
76	11	3	0.000	.
78	11	3	.	.
2	1	6	0.000	.
10	1	6	0.000	.
12	1	6	0.000	.
18	1	6	0.000	.
19	1	6	.	.
7	2	6	0.000	.
9	2	6	.	.
11	2	6	0.000	.
13	2	6	0.003	.
14	2	6	0.000	.
48	3	6	0.001	.
56	3	6	0.000	.
57	3	6	0.000	.
58	3	6	0.006	.
59	3	6	0.005	.
44	4	6	0.007	.
45	4	6	0.007	.
51	4	6	0.004	.
52	4	6	0.000	.
54	4	6	0.001	.
23	5	6	.	.
32	5	6	0.000	.
35	5	6	0.007	.
37	5	6	0.000	.
40	5	6	0.000	.
3	6	6	0.007	.
20	6	6	0.020	.

41	6	6	0.016	.
47	6	6	0.000	.
55	6	6	0.000	.
53	6	6	0.000	.
21	7	6	0.000	.
24	7	6	0.000	.
26	7	6	.	.
31	7	6	.	.
36	7	6	0.000	.
25	8	6	.	.
29	8	6	0.000	.
30	8	6	0.000	.
33	8	6	0.000	.
89	8	6	0.011	.
81	9	6	0.002	.
82	9	6	0.001	.
64	9	6	0.042	.
88	9	6	0.026	.
91	9	6	0.000	.
74	10	6	0.000	.
66	10	6	0.000	.
71	10	6	.	.
77	10	6	0.000	.
79	10	6	0.024	.
65	11	6	.	.
69	11	6	0.000	.
70	11	6	0.000	.
76	11	6	0.000	.
78	11	6	.	.
2	1	9	0.000	48
10	1	9	0.000	59
12	1	9	0.000	47.8
18	1	9	0.000	46.6
19	1	9	.	.
7	2	9	0.000	39
9	2	9	.	.
11	2	9	0.000	63
13	2	9	.	.
14	2	9	0.000	53
48	3	9	0.000	53
56	3	9	0.005	44.4
57	3	9	0.005	44.4
58	3	9	0.001	48
59	3	9	.	.
44	4	9	0.012	39.2
45	4	9	0.000	51.5
51	4	9	0.007	39.4
52	4	9	0.000	61
54	4	9	0.001	38.4
23	5	9	.	.
32	5	9	0.000	49.8
35	5	9	0.000	53
37	5	9	0.000	48.4
40	5	9	0.000	61.5
3	6	9	0.011	44.8
20	6	9	0.008	38.6
41	6	9	0.000	55
47	6	9	0.000	64
55	6	9	0.000	48.4
53	6	9	.	.
21	7	9	.	.
24	7	9	0.001	63
26	7	9	.	.
31	7	9	.	.
36	7	9	0.006	60
25	8	9	.	.
29	8	9	0.000	59
30	8	9	0.001	54
33	8	9	0.000	60.5
89	8	9	0.011	59.5
81	9	9	0.000	43.4
82	9	9	0.000	57.5
64	9	9	0.018	42.6
88	9	9	0.018	53
91	9	9	0.000	52.5
74	10	9	0.000	57.5
66	10	9	0.000	62.5
71	10	9	.	.
77	10	9	0.005	39
79	10	9	0.022	43.6
65	11	9	.	.
69	11	9	0.004	52
70	11	9	0.000	54
76	11	9	0.000	33
78	11	9	.	.

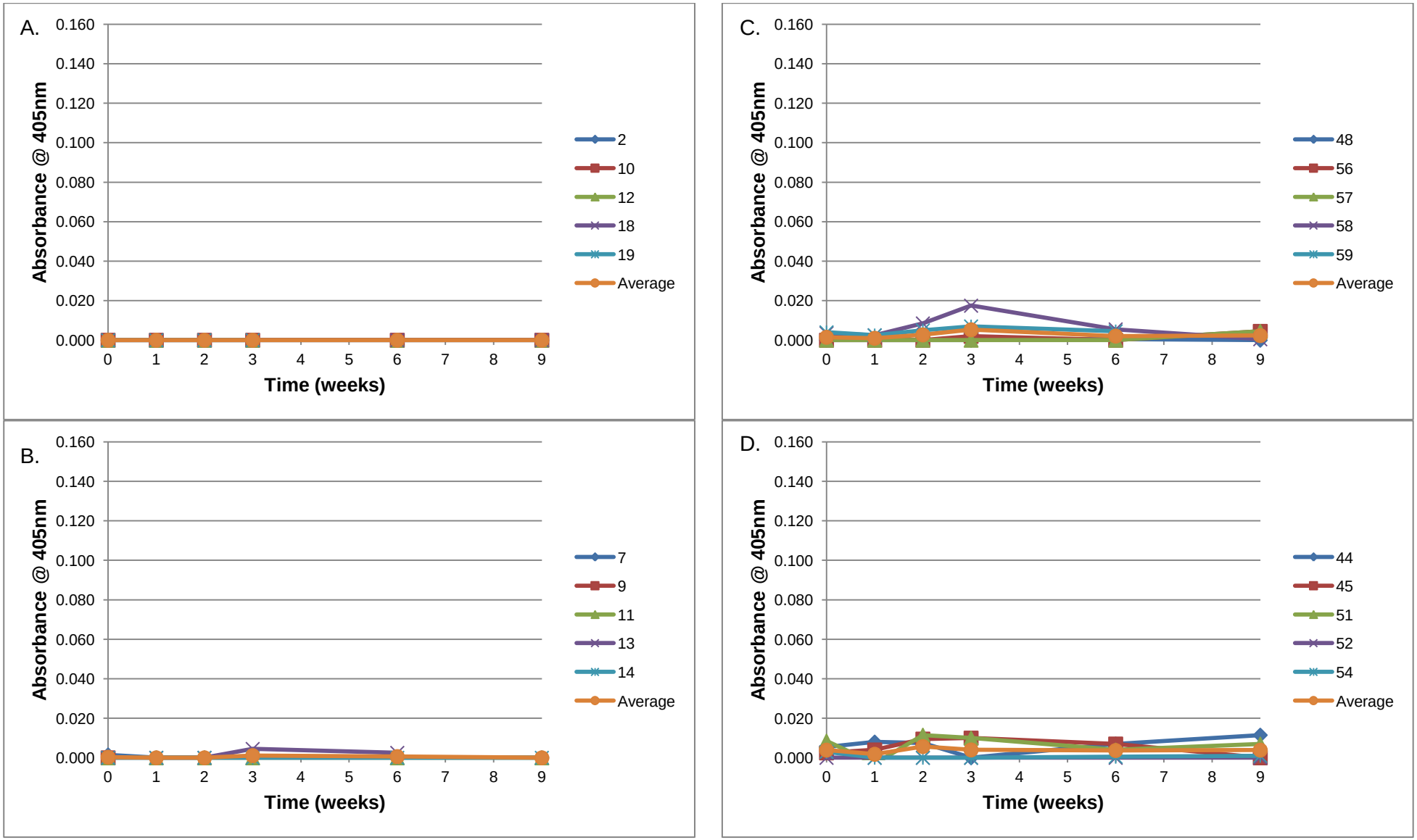

Figure C.4: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^9 c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. 10^{10} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, C. 10^{11} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, D. Received a 10^{12} c.f.u./ml dose of the VR1012 mucosal DNA vaccine.

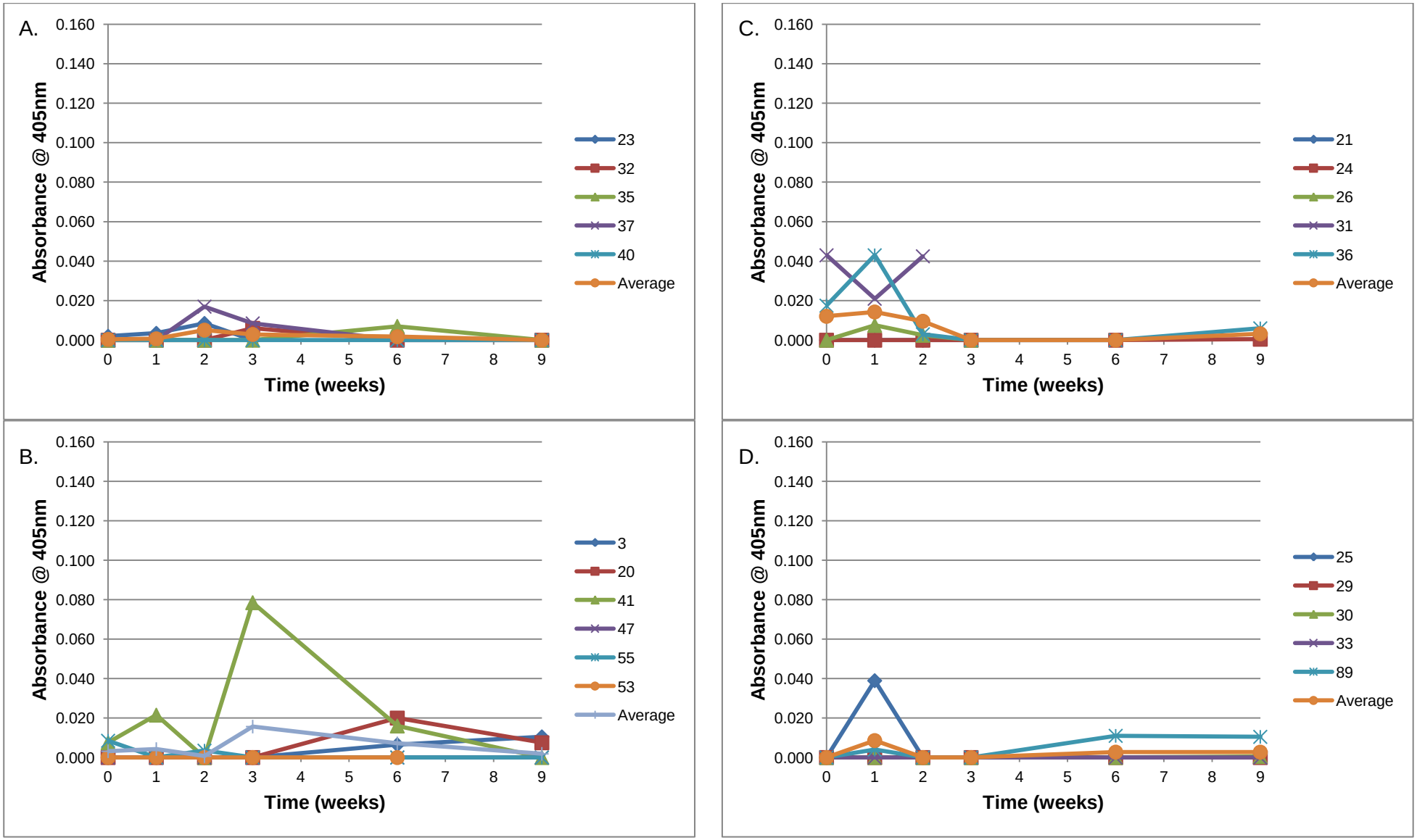

Figure C.5: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{13} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. Control group, C. 10^{9} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, D. Received a 10^{10} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

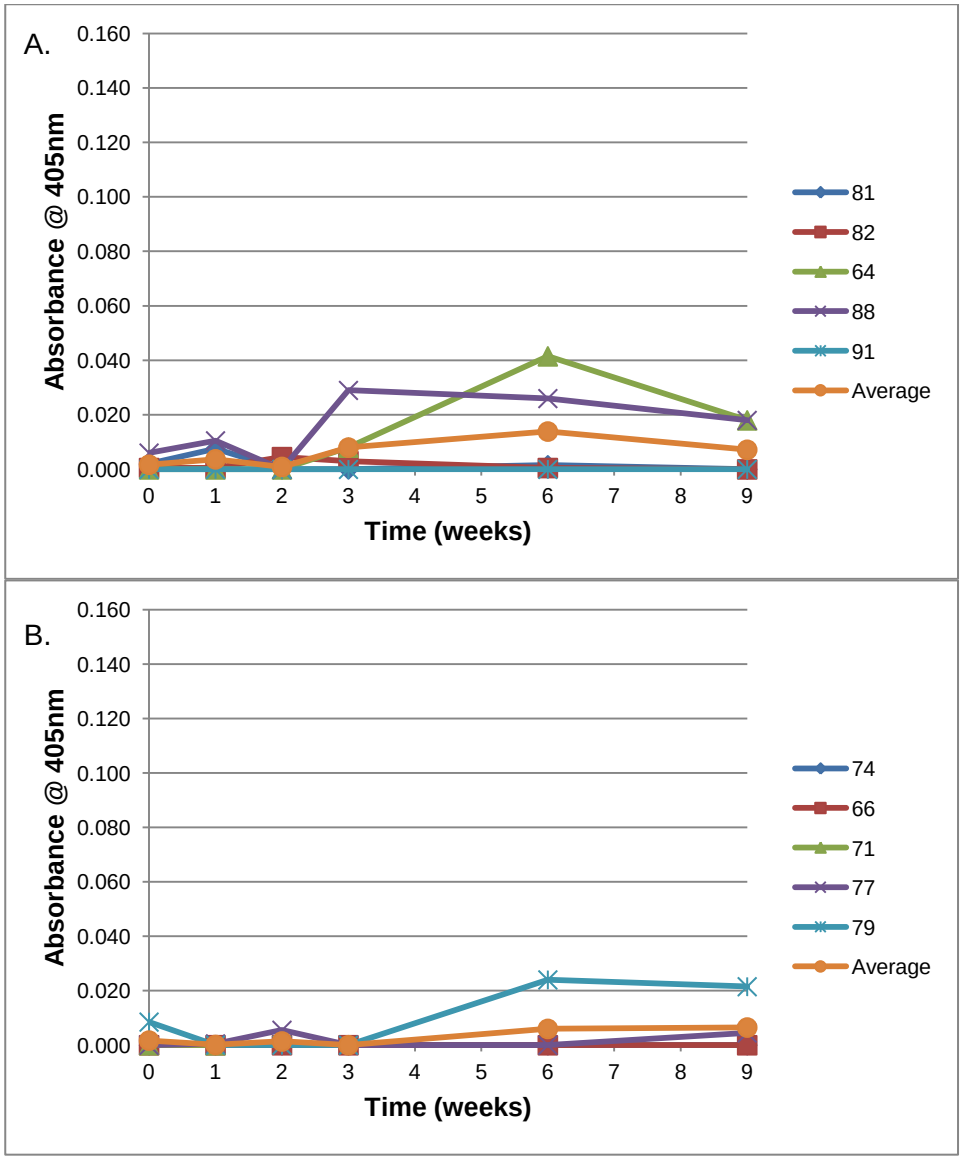

Figure C.6: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{11} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, B. 10^{12} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, C. 10^{13} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

Mucosal immune response of ostriches against *Mycoplasma struthionis*

The mucosal immune response of the ostriches against the OppA protein was evaluated using ELISA as described in section 5.2.3., using the secondary antibody rabbit anti-ostric IgA protein 2. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – OppA – IgA Protein 2
Kromme Rhee
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	329	0.039			
Treatment	10	0.004	0.00	3.27	0.0005
Time	5	0.001	0.00	0.95	0.449
Treatment x Time	50	0.004	0.00	0.65	0.9675
Residual	264	0.031	0.00		

Grand mean = 0.005 R-squared = 0.2090 C.V. = 236.80%

LSD for Treatment = 0.0055 S.E.D = 0.0028 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00012

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
7	0.01	199.9	1	VR1020 10^9
10	0.01	151.9	2	VR1020 10^12
1	0.01	150.8	3	VR1012 10^9
9	0.01	183.5	4	VR1020 10^11
4	0.01	182.5	5	VR1012 10^12
3	0.00	168.7	6	VR1012 10^11
11	0.00	253.1	7	VR1020 10^13
6	0.00	218.7	8	Control
2	0.00	203.4	9	VR1012 10^10
5	0.00	-9	10	VR1012 10^13
8	0.00	-9	11	VR1020 10^10

LSD for Time = 0.0041 S.E.D = 0.0021 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00012

Time
Averages

Level	--- Y ---	Cv	Rank	Week
1	0.01	247.4	1	0
5	0.01	189.4	2	6
6	0.00	219.5	3	9
4	0.00	233.7	4	3
2	0.00	210.1	5	1
3	0.00	282.3	6	2

LSD for Treatment*Time = 0.0135 S.E.D = 0.0069 r = 5
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00012

Two-way table for Treatment*Time, n=5

	1	2	3	4	5	6
1	0.010	0.004	0.008	0.010	0.006	0.004
2	0.004	0.000	0.000	0.004	0.004	0.000
3	0.004	0.004	0.004	0.008	0.004	0.000
4	0.002	0.002	0.002	0.006	0.012	0.008
5	0.000	0.000	0.000	0.000	0.000	0.000
6	0.004	0.002	0.000	0.000	0.006	0.004
7	0.028	0.010	0.010	0.008	0.014	0.002
8	0.000	0.000	0.000	0.000	0.000	0.000
9	0.010	0.010	0.002	0.002	0.002	0.008
10	0.012	0.004	0.002	0.004	0.010	0.016
11	0.002	0.002	0.008	0.000	0.004	0.006

The following table contains the input data for the ANOVA and is arranged in two columns with the ostrich identification number, treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The treatments consist of VR1012 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (1), $1x10^{10}$ c.f.u./ml (2), $1x10^{11}$ c.f.u./ml (3), $1x10^{12}$ c.f.u./ml (4) and $1x10^{13}$ c.f.u./ml (5); *Salmonella enterica* serovar *typhimurium* SL3261 control (6), VR1020 naked DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (7), $1x10^{10}$ c.f.u./ml (8), $1x10^{11}$ c.f.u./ml (9), $1x10^{12}$ c.f.u./ml (10) and $1x10^{13}$ c.f.u./ml (11). Following the data are the graphs drawn for each ostrich depicting the absorbance measured over time.

Ostrich	Trt	Time	Resp	Weight
2	1	0	0.000	44.6
10	1	0	0.031	47.6
12	1	0	0.017	50.4
18	1	0	0.000	45.2
19	1	0	0.000	47.8
7	2	0	0.000	44.2
9	2	0	0.000	46.8
11	2	0	0.000	48.4
13	2	0	0.005	50
14	2	0	0.007	47
48	3	0	0.001	48.6
56	3	0	0.000	48
57	3	0	0.000	47.6
58	3	0	0.011	47.6
59	3	0	0.012	46.2
44	4	0	0.003	48.2
45	4	0	0.013	46.6
51	4	0	0.000	45.6
52	4	0	0.000	46.8
54	4	0	0.000	51
23	5	0	0.000	51.6
32	5	0	0.000	46.6
35	5	0	0.000	50.4
37	5	0	0.000	49.8
40	5	0	0.000	47.2
3	6	0	0.019	52.4
20	6	0	0.000	52.4
41	6	0	0.000	51.8
47	6	0	0.000	54.8
55	6	0	0.000	55.8
53	6	0	0.008	55.4
21	7	0	0.000	48.8
24	7	0	0.000	50
26	7	0	0.003	52.8
31	7	0	0.029	47
36	7	0	0.107	51.2
25	8	0	0.000	48.2
29	8	0	0.000	46.8
30	8	0	0.000	50
33	8	0	0.000	53.8
89	8	0	0.000	53.2
81	9	0	0.000	44
82	9	0	0.031	50.8
64	9	0	0.002	54
88	9	0	0.024	50.6
91	9	0	0.000	50.8
74	10	0	0.000	55.6
66	10	0	0.000	48.2
71	10	0	0.000	44.8
77	10	0	0.028	49
79	10	0	0.030	44.6
65	11	0	0.000	50.4
69	11	0	0.000	50.6
70	11	0	0.000	47.6
76	11	0	0.000	44
78	11	0	0.011	46.2
2	1	1	0.000	.
10	1	1	0.009	.
12	1	1	0.014	.
18	1	1	0.000	.
19	1	1	0.000	.
7	2	1	0.000	.
9	2	1	.	.
11	2	1	0.000	.
13	2	1	0.002	.
14	2	1	0.000	.
48	3	1	0.000	.
56	3	1	0.000	.
57	3	1	0.009	.
58	3	1	0.000	.
59	3	1	0.005	.
44	4	1	0.001	.
45	4	1	0.011	.
51	4	1	0.000	.
52	4	1	0.000	.
54	4	1	0.000	.
23	5	1	0.000	.
32	5	1	0.000	.
35	5	1	0.000	.
37	5	1	0.000	.
40	5	1	0.000	.
3	6	1	0.006	.
20	6	1	0.000	.
41	6	1	0.000	.
47	6	1	0.000	.
55	6	1	0.000	.
53	6	1	0.008	.
21	7	1	0.021	.
24	7	1	0.000	.
26	7	1	0.004	.
31	7	1	0.014	.
36	7	1	0.024	.
25	8	1	0.000	.
29	8	1	0.000	.
30	8	1	0.000	.
33	8	1	0.000	.
89	8	1	0.000	.
81	9	1	0.000	.
82	9	1	0.042	.
64	9	1	0.000	.
88	9	1	0.007	.
91	9	1	0.000	.
74	10	1	0.000	.
66	10	1	0.000	.
71	10	1	0.000	.
77	10	1	0.009	.
79	10	1	0.012	.
65	11	1	.	.
69	11	1	0.000	.
70	11	1	0.003	.
76	11	1	0.000	.
78	11	1	0.009	.
2	1	2	0.005	41.8
10	1	2	0.011	51.5
12	1	2	0.019	47
18	1	2	0.000	40.2
19	1	2	0.000	36.2
7	2	2	0.000	43.2
9	2	2	.	.
11	2	2	0.000	44.4
13	2	2	0.004	44.4
14	2	2	0.000	40.6
48	3	2	0.000	47
56	3	2	0.000	40.6
57	3	2	0.000	44.2

58	3	2	0.009	36
59	3	2	0.010	36.6
44	4	2	0.000	40
45	4	2	0.009	39.8
51	4	2	0.000	40
52	4	2	0.000	58.5
54	4	2	0.000	42
23	5	2	0.000	47.6
32	5	2	0.000	45
35	5	2	0.000	47.8
37	5	2	0.000	47
40	5	2	0.000	41.4
3	6	2	0.000	53
20	6	2	0.000	47.4
41	6	2	0.000	41.2
47	6	2	0.000	47
55	6	2	0.000	50.5
53	6	2	0.023	47.2
21	7	2	0.000	51
24	7	2	0.000	55.5
26	7	2	0.004	42.8
31	7	2	0.047	35
36	7	2	0.000	52.5
25	8	2	0.000	34.6
29	8	2	0.000	45
30	8	2	0.000	48.2
33	8	2	0.000	51
89	8	2	0.000	54
81	9	2	0.000	46.8
82	9	2	0.010	46.2
64	9	2	0.000	45.8
88	9	2	0.000	52.5
91	9	2	0.000	37.2
74	10	2	0.000	49
66	10	2	0.000	42.6
71	10	2	.	.
77	10	2	0.004	39.2
79	10	2	0.007	40.4
65	11	2	.	.
69	11	2	0.041	40.6
70	11	2	0.000	41.4
76	11	2	0.000	36.2
78	11	2	.	.
2	1	3	0.009	.
10	1	3	0.039	.
12	1	3	0.000	.
18	1	3	0.000	.
19	1	3	0.000	.
7	2	3	0.000	.
9	2	3	.	.
11	2	3	0.000	.
13	2	3	0.009	.
14	2	3	0.009	.
48	3	3	0.000	.
56	3	3	0.000	.
57	3	3	0.016	.
58	3	3	0.000	.
59	3	3	0.015	.
44	4	3	0.014	.
45	4	3	0.015	.
51	4	3	0.002	.
52	4	3	0.000	.
54	4	3	0.000	.

23	5	3	0.000	.
32	5	3	0.000	.
35	5	3	0.000	.
37	5	3	0.000	.
40	5	3	0.000	.
3	6	3	0.000	.
20	6	3	0.000	.
41	6	3	0.000	.
47	6	3	0.000	.
55	6	3	0.000	.
53	6	3	0.000	.
21	7	3	0.001	.
24	7	3	0.043	.
26	7	3	0.003	.
31	7	3	.	.
36	7	3	0.000	.
25	8	3	0.000	.
29	8	3	0.000	.
30	8	3	0.000	.
33	8	3	0.000	.
89	8	3	0.000	.
81	9	3	0.000	.
82	9	3	0.006	.
64	9	3	0.000	.
88	9	3	0.000	.
91	9	3	0.001	.
74	10	3	0.000	.
66	10	3	0.000	.
71	10	3	.	.
77	10	3	0.007	.
79	10	3	0.013	.
65	11	3	.	.
69	11	3	0.000	.
70	11	3	0.000	.
76	11	3	0.000	.
78	11	3	.	.
2	1	6	0.018	.
10	1	6	0.007	.
12	1	6	0.000	.
18	1	6	0.000	.
19	1	6	.	.
7	2	6	0.000	.
9	2	6	.	.
11	2	6	0.000	.
13	2	6	0.005	.
14	2	6	0.012	.
48	3	6	0.000	.
56	3	6	0.000	.
57	3	6	0.000	.
58	3	6	0.004	.
59	3	6	0.015	.
44	4	6	0.025	.
45	4	6	0.030	.
51	4	6	0.000	.
52	4	6	0.000	.
54	4	6	0.000	.
23	5	6	.	.
32	5	6	0.000	.
35	5	6	0.000	.
37	5	6	0.000	.
40	5	6	0.000	.
3	6	6	0.000	.
20	6	6	0.017	.

41	6	6	0.000	.
47	6	6	0.000	.
55	6	6	0.014	.
53	6	6	0.011	.
21	7	6	0.021	.
24	7	6	0.054	.
26	7	6	.	.
31	7	6	.	.
36	7	6	0.000	.
25	8	6	.	.
29	8	6	0.000	.
30	8	6	0.000	.
33	8	6	0.000	.
89	8	6	0.000	.
81	9	6	0.003	.
82	9	6	0.000	.
64	9	6	0.009	.
88	9	6	0.004	.
91	9	6	0.000	.
74	10	6	0.000	.
66	10	6	0.000	.
71	10	6	.	.
77	10	6	0.021	.
79	10	6	0.028	.
65	11	6	.	.
69	11	6	0.000	.
70	11	6	0.006	.
76	11	6	0.007	.
78	11	6	.	.
2	1	9	0.000	48
10	1	9	0.022	59
12	1	9	0.000	47.8
18	1	9	0.000	46.6
19	1	9	.	.
7	2	9	0.000	39
9	2	9	.	.
11	2	9	0.000	63
13	2	9	.	.
14	2	9	0.004	53
48	3	9	0.000	53
56	3	9	0.000	44.4
57	3	9	0.000	44.4
58	3	9	0.002	48
59	3	9	.	.
44	4	9	0.030	39.2
45	4	9	0.000	51.5
51	4	9	0.007	39.4
52	4	9	0.000	61
54	4	9	0.000	38.4
23	5	9	.	.
32	5	9	0.000	49.8
35	5	9	0.000	53
37	5	9	0.000	48.4
40	5	9	0.000	61.5
3	6	9	0.005	44.8
20	6	9	0.000	38.6
41	6	9	0.000	55
47	6	9	0.000	64
55	6	9	0.011	48.4
53	6	9	.	.
21	7	9	.	.
24	7	9	0.004	63
26	7	9	.	.
31	7	9	.	.
36	7	9	0.007	60
25	8	9	.	.
29	8	9	0.000	59
30	8	9	0.000	54
33	8	9	0.000	60.5
89	8	9	0.000	59.5
81	9	9	0.000	43.4
82	9	9	0.020	57.5
64	9	9	0.024	42.6
88	9	9	0.000	53
91	9	9	0.000	52.5
74	10	9	0.029	57.5
66	10	9	0.000	62.5
71	10	9	.	.
77	10	9	0.035	39
79	10	9	0.011	43.6
65	11	9	.	.
69	11	9	0.000	52
70	11	9	0.028	54
76	11	9	0.001	33
78	11	9	.	.

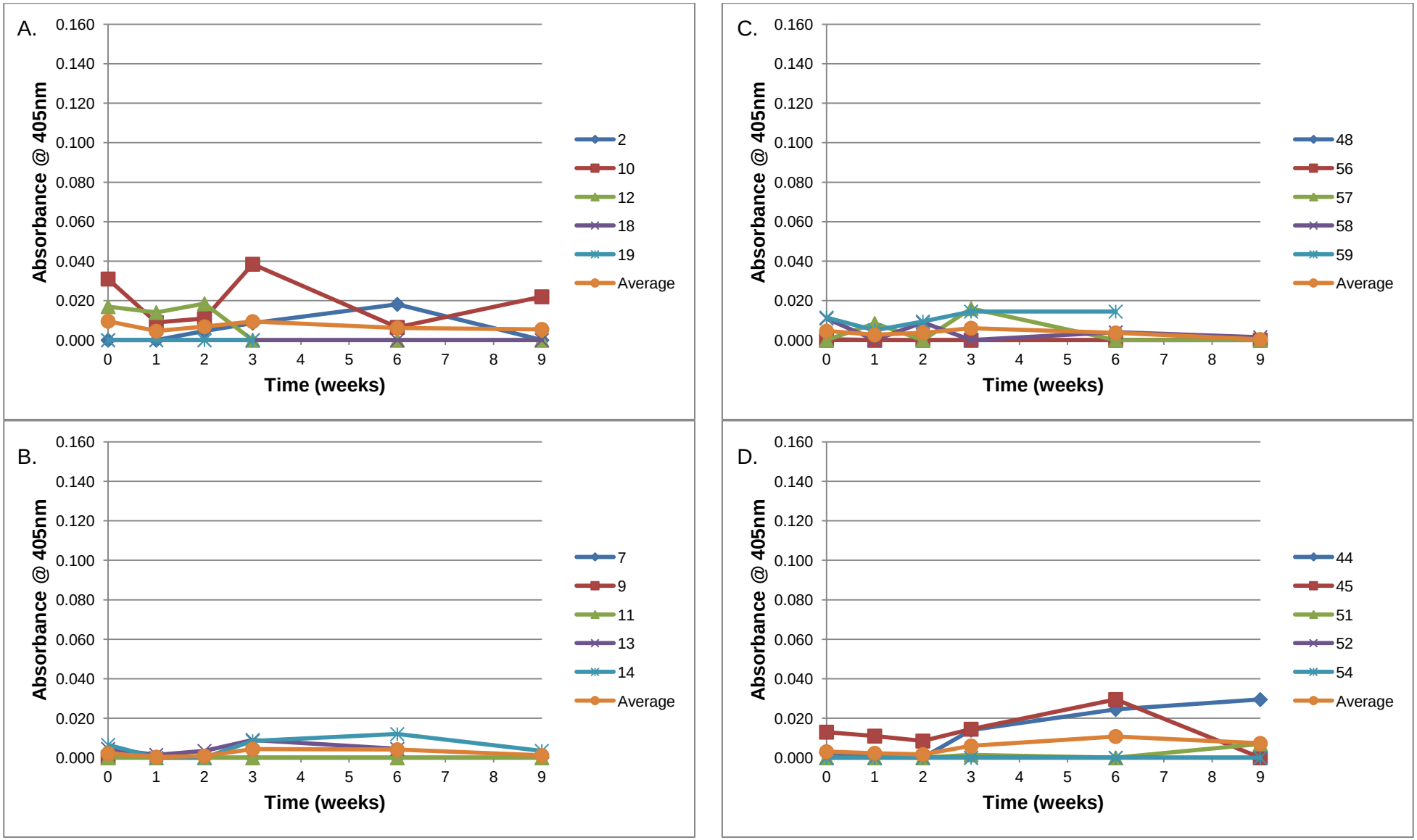

Figure C.7: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^9 c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. 10^{10} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, C. 10^{11} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, D. Received a 10^{12} c.f.u./ml dose of the VR1012 mucosal DNA vaccine.

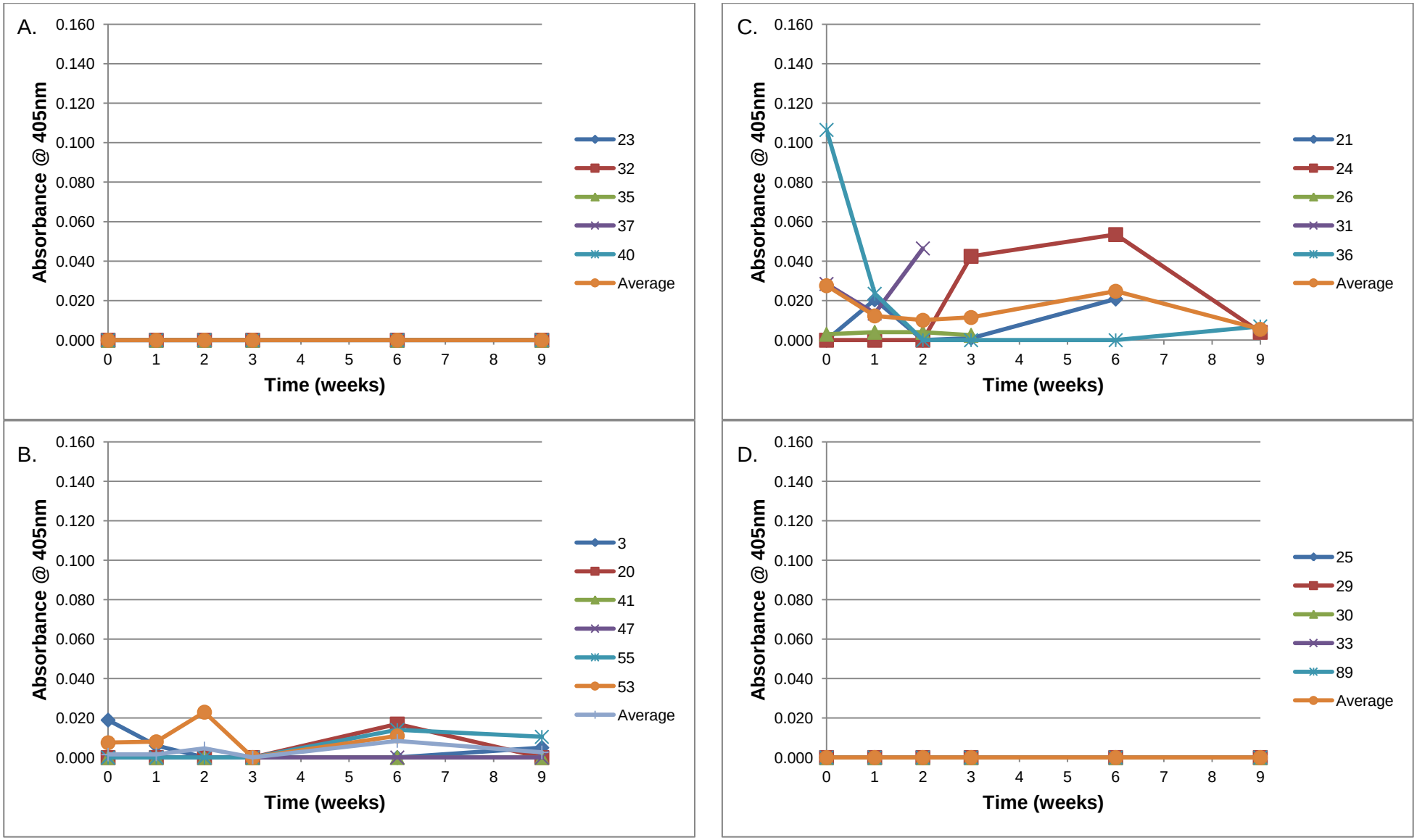

Figure C.8: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{13} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. Control group, C. 10^{9} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, D. Received a 10^{10} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

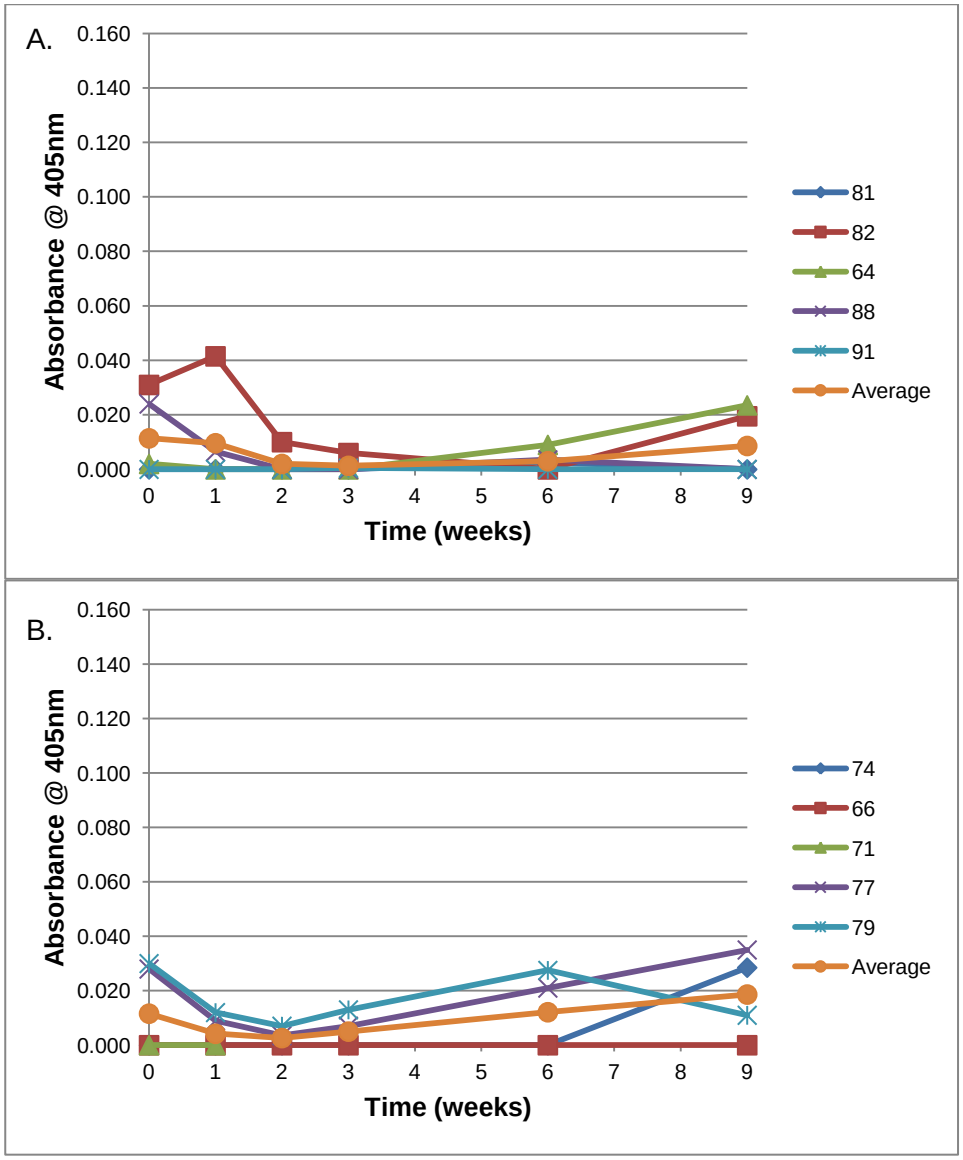

Figure C.9: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{11} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, B. 10^{12} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, C. 10^{13} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

Humoral immune response of ostriches against *Salmonella enterica* serovar *typhimurium* SL3261 LPS

The humoral immune response of the ostriches against the LPS of *Salmonella enterica* serovar *typhimurium* SL3261 was evaluated using ELISA as described in section 5.2.4. The data was analysed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.). FAC_A represents the treatment and FAC_B represent the time in weeks.

A N A L Y S I S O F V A R I A N C E
Serum - LPS
Kromme Rhee
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	329	146.639			
Treatment	10	30.985	3.099	9.72	0.0000
Time	5	10.501	2.100	6.59	0.0000
Treatment x Time	50	20.993	0.420	1.32	0.0887
Residual	264	84.159	0.319		

Grand mean = 1.035 R-squared = 0.4261 C.V. = 54.54%

LSD for Treatment = 0.2870 S.E.D = 0.1458 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.31878

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
9	1.54	33.8	1	VR1020 10^11
6	1.38	43.6	2	Control
8	1.38	49	3	VR1020 10^10
7	1.21	60.1	4	VR1020 10^9
10	1.19	57.6	5	VR1020 10^12
5	0.94	54.8	6	VR1012 10^13
3	0.93	66.8	7	VR1012 10^11
11	0.82	79.5	8	VR1020 10^13
4	0.72	71.9	9	VR1012 10^12
1	0.7	67.2	10	VR1012 10^9
2	0.58	100.6	11	VR1012 10^10

LSD for Time = 0.2120 S.E.D = 0.1077 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.31878

Time
Averages

Level	--- Y ---	Cv	Rank	Week
2	1.24	54.5	1	1
1	1.21	49.2	2	0
4	1.11	64.6	3	3
3	1.02	63.7	4	2
5	0.89	73.7	5	6
6	0.74	79.1	6	9

LSD for Treatment*Time = 0.7031 S.E.D = 0.3571 r = 5
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.31878

Two-way table for Treatment*Time, n=5

	1	2	3	4	5	6
1	0.688	0.500	0.466	0.898	0.814	0.846
2	0.458	0.500	0.332	0.454	0.794	0.928
3	1.112	1.006	0.882	0.762	1.040	0.748
4	0.820	0.788	0.612	0.962	0.650	0.516
5	0.942	1.352	1.032	0.828	0.818	0.674
6	1.394	1.402	1.456	1.858	1.292	0.902
7	1.452	1.864	1.534	1.112	0.832	0.454
8	1.820	1.916	1.618	1.578	0.596	0.736
9	1.558	1.710	1.256	1.920	1.570	1.220
10	1.568	1.612	1.272	1.282	0.876	0.542
11	1.488	1.008	0.800	0.604	0.464	0.532

The following table contains the input data for the ANOVA and is arranged in two columns with the ostrich identification number, treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The treatments consist of VR1012 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (1), $1x10^{10}$ c.f.u./ml (2), $1x10^{11}$ c.f.u./ml (3), $1x10^{12}$ c.f.u./ml (4) and $1x10^{13}$ c.f.u./ml (5); *Salmonella enterica* serovar *typhimurium* SL3261 control (6), VR1020 naked DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (7), $1x10^{10}$ c.f.u./ml (8), $1x10^{11}$ c.f.u./ml (9), $1x10^{12}$ c.f.u./ml (10) and $1x10^{13}$ c.f.u./ml (11). Following the data are the graphs drawn for each ostrich depicting the absorbance measured over time.

Ostrich	Trt	Time	Resp	Weight
2	1	0	0.837	44.6
10	1	0	0.528	47.6
12	1	0	0.713	50.4
18	1	0	0.664	45.2
19	1	0	0.703	47.8
7	2	0	0.580	44.2
9	2	0	0.000	46.8
11	2	0	0.624	48.4
13	2	0	0.451	50
14	2	0	0.638	47
48	3	0	0.313	48.6
56	3	0	2.641	48
57	3	0	1.290	47.6
58	3	0	0.898	47.6
59	3	0	0.418	46.2
44	4	0	0.606	48.2
45	4	0	1.425	46.6
51	4	0	0.620	45.6
52	4	0	1.032	46.8
54	4	0	0.413	51
23	5	0	1.251	51.6
32	5	0	1.382	46.6
35	5	0	0.804	50.4
37	5	0	0.497	49.8
40	5	0	0.775	47.2
3	6	0	1.204	52.4
20	6	0	1.544	52.4
41	6	0	1.009	51.8
47	6	0	1.060	54.8
55	6	0	2.160	55.8
53	6	0	1.504	55.4
21	7	0	1.698	48.8
24	7	0	1.284	50
26	7	0	1.735	52.8
31	7	0	0.976	47
36	7	0	1.558	51.2
25	8	0	2.157	48.2
29	8	0	1.545	46.8
30	8	0	2.074	50
33	8	0	2.206	53.8
89	8	0	1.111	53.2
81	9	0	1.678	44
82	9	0	2.318	50.8
64	9	0	1.254	54
88	9	0	1.447	50.6
91	9	0	1.094	50.8
74	10	0	1.218	55.6
66	10	0	1.445	48.2
71	10	0	2.549	44.8
77	10	0	1.472	49
79	10	0	1.146	44.6
65	11	0	1.389	50.4
69	11	0	1.706	50.6
70	11	0	1.109	47.6
76	11	0	1.421	44
78	11	0	1.807	46.2
2	1	1	0.380	.
10	1	1	0.401	.
12	1	1	0.791	.
18	1	1	0.385	.
19	1	1	0.538	.
7	2	1	0.837	.
9	2	1	.	.
11	2	1	0.477	.
13	2	1	0.382	.
14	2	1	0.803	.
48	3	1	0.556	.
56	3	1	1.982	.
57	3	1	1.305	.
58	3	1	0.784	.
59	3	1	0.399	.
44	4	1	0.353	.
45	4	1	2.082	.
51	4	1	0.464	.
52	4	1	0.634	.
54	4	1	0.423	.
23	5	1	1.003	.
32	5	1	2.559	.
35	5	1	1.229	.
37	5	1	0.859	.
40	5	1	1.105	.
3	6	1	1.183	.
20	6	1	1.354	.
41	6	1	0.795	.
47	6	1	1.636	.
55	6	1	2.044	.
53	6	1	2.004	.
21	7	1	2.111	.
24	7	1	1.884	.
26	7	1	1.956	.
31	7	1	1.731	.
36	7	1	1.637	.
25	8	1	1.523	.
29	8	1	2.184	.
30	8	1	1.554	.
33	8	1	2.612	.
89	8	1	1.718	.
81	9	1	1.182	.
82	9	1	2.295	.
64	9	1	1.866	.
88	9	1	1.612	.
91	9	1	1.594	.
74	10	1	1.008	.
66	10	1	1.970	.
71	10	1	1.873	.
77	10	1	1.862	.
79	10	1	1.349	.
65	11	1	.	.
69	11	1	1.863	.
70	11	1	0.745	.
76	11	1	1.133	.
78	11	1	1.304	.
2	1	2	0.661	41.8
10	1	2	0.158	51.5
12	1	2	0.823	47
18	1	2	0.406	40.2
19	1	2	0.282	36.2
7	2	2	0.000	43.2
9	2	2	.	.
11	2	2	0.422	44.4
13	2	2	0.279	44.4
14	2	2	0.961	40.6
48	3	2	0.443	47
56	3	2	1.688	40.6
57	3	2	1.027	44.2

58	3	2	0.876	36
59	3	2	0.366	36.6
44	4	2	0.293	40
45	4	2	1.416	39.8
51	4	2	0.503	40
52	4	2	0.532	58.5
54	4	2	0.320	42
23	5	2	1.000	47.6
32	5	2	1.839	45
35	5	2	0.873	47.8
37	5	2	0.650	47
40	5	2	0.801	41.4
3	6	2	1.234	53
20	6	2	1.446	47.4
41	6	2	0.968	41.2
47	6	2	1.755	47
55	6	2	1.871	50.5
53	6	2	1.643	47.2
21	7	2	2.094	51
24	7	2	2.313	55.5
26	7	2	1.659	42.8
31	7	2	0.558	35
36	7	2	1.048	52.5
25	8	2	1.349	34.6
29	8	2	1.925	45
30	8	2	1.603	48.2
33	8	2	2.304	51
89	8	2	0.912	54
81	9	2	1.172	46.8
82	9	2	2.122	46.2
64	9	2	1.074	45.8
88	9	2	0.994	52.5
91	9	2	0.928	37.2
74	10	2	1.431	49
66	10	2	1.590	42.6
71	10	2	.	.
77	10	2	1.804	39.2
79	10	2	1.537	40.4
65	11	2	.	.
69	11	2	1.893	40.6
70	11	2	0.999	41.4
76	11	2	1.109	36.2
78	11	2	.	.
2	1	3	0.905	.
10	1	3	0.678	.
12	1	3	0.992	.
18	1	3	1.643	.
19	1	3	0.265	.
7	2	3	0.663	.
9	2	3	.	.
11	2	3	0.369	.
13	2	3	0.475	.
14	2	3	0.759	.
48	3	3	0.330	.
56	3	3	0.673	.
57	3	3	1.482	.
58	3	3	0.944	.
59	3	3	0.394	.
44	4	3	0.363	.
45	4	3	1.955	.
51	4	3	1.371	.
52	4	3	0.848	.
54	4	3	0.265	.
23	5	3	0.843	.
32	5	3	1.265	.
35	5	3	0.595	.
37	5	3	0.568	.
40	5	3	0.857	.
3	6	3	2.427	.
20	6	3	1.862	.
41	6	3	0.494	.
47	6	3	1.802	.
55	6	3	2.713	.
53	6	3	1.451	.
21	7	3	1.593	.
24	7	3	1.493	.
26	7	3	1.632	.
31	7	3	.	.
36	7	3	0.848	.
25	8	3	1.723	.
29	8	3	1.567	.
30	8	3	1.604	.
33	8	3	1.859	.
89	8	3	1.141	.
81	9	3	0.957	.
82	9	3	1.619	.
64	9	3	2.491	.
88	9	3	2.014	.
91	9	3	2.515	.
74	10	3	1.568	.
66	10	3	0.912	.
71	10	3	.	.
77	10	3	2.363	.
79	10	3	1.569	.
65	11	3	.	.
69	11	3	1.324	.
70	11	3	0.789	.
76	11	3	0.910	.
78	11	3	.	.
2	1	6	0.562	.
10	1	6	0.759	.
12	1	6	0.825	.
18	1	6	1.916	.
19	1	6	.	.
7	2	6	0.326	.
9	2	6	.	.
11	2	6	0.601	.
13	2	6	0.830	.
14	2	6	2.212	.
48	3	6	0.347	.
56	3	6	1.103	.
57	3	6	1.996	.
58	3	6	1.277	.
59	3	6	0.473	.
44	4	6	0.539	.
45	4	6	1.375	.
51	4	6	0.348	.
52	4	6	0.767	.
54	4	6	0.206	.
23	5	6	.	.
32	5	6	1.279	.
35	5	6	1.284	.
37	5	6	0.724	.
40	5	6	0.808	.
3	6	6	1.050	.
20	6	6	1.124	.

41	6	6	0.338	.
47	6	6	1.467	.
55	6	6	2.478	.
53	6	6	0.433	.
21	7	6	1.237	.
24	7	6	1.222	.
26	7	6	.	.
31	7	6	.	.
36	7	6	1.696	.
25	8	6	.	.
29	8	6	0.990	.
30	8	6	0.403	.
33	8	6	1.008	.
89	8	6	0.577	.
81	9	6	1.736	.
82	9	6	0.888	.
64	9	6	1.173	.
88	9	6	2.052	.
91	9	6	2.002	.
74	10	6	0.557	.
66	10	6	0.734	.
71	10	6	.	.
77	10	6	1.519	.
79	10	6	1.569	.
65	11	6	.	.
69	11	6	1.206	.
70	11	6	0.615	.
76	11	6	0.494	.
78	11	6	.	.
2	1	9	0.726	48
10	1	9	0.403	59
12	1	9	1.182	47.8
18	1	9	1.924	46.6
19	1	9	.	.
7	2	9	0.927	39
9	2	9	.	.
11	2	9	1.407	63
13	2	9	.	.
14	2	9	2.303	53
48	3	9	0.388	53
56	3	9	0.627	44.4
57	3	9	1.540	44.4
58	3	9	1.176	48
59	3	9	.	.
44	4	9	0.288	39.2
45	4	9	1.099	51.5
51	4	9	0.206	39.4
52	4	9	0.805	61
54	4	9	0.174	38.4
23	5	9	.	.
32	5	9	1.623	49.8
35	5	9	0.937	53
37	5	9	0.522	48.4
40	5	9	0.291	61.5
3	6	9	1.017	44.8
20	6	9	1.130	38.6
41	6	9	0.404	55
47	6	9	1.264	64
55	6	9	0.698	48.4
53	6	9	-	.
21	7	9	-	.
24	7	9	0.894	63
26	7	9	.	.
31	7	9	.	.
36	7	9	1.375	60
25	8	9	.	.
29	8	9	0.519	59
30	8	9	0.790	54
33	8	9	1.526	60.5
89	8	9	0.837	59.5
81	9	9	1.162	43.4
82	9	9	1.600	57.5
64	9	9	1.536	42.6
88	9	9	0.544	53
91	9	9	1.256	52.5
74	10	9	0.832	57.5
66	10	9	0.610	62.5
71	10	9	.	.
77	10	9	0.472	39
79	10	9	0.800	43.6
65	11	9	.	.
69	11	9	1.379	52
70	11	9	0.631	54
76	11	9	0.651	33
78	11	9	.	.

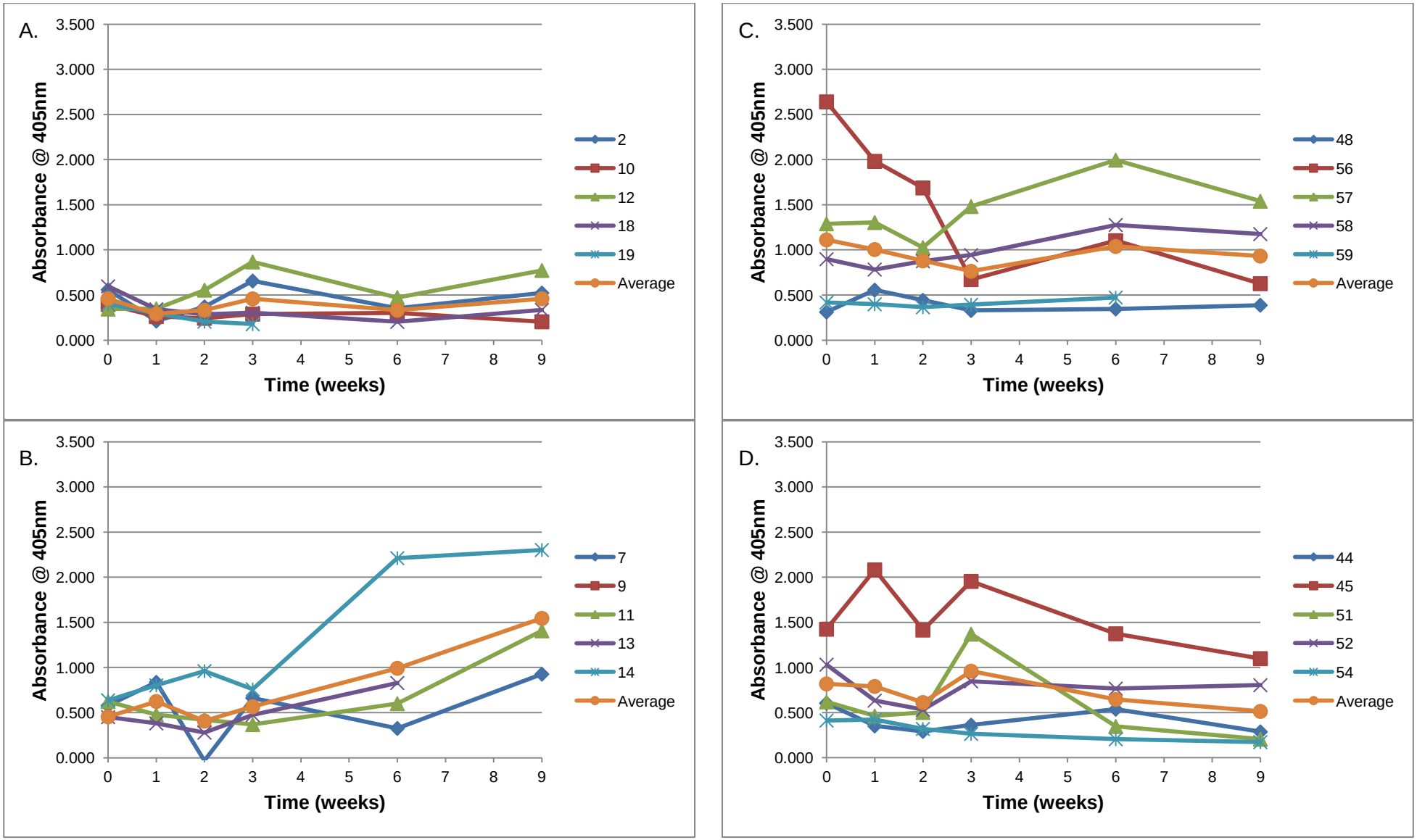

Figure C.10: Humoral immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^9 c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. 10^{10} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, C. 10^{11} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, D. Received a 10^{12} c.f.u./ml dose of the VR1012 mucosal DNA vaccine.

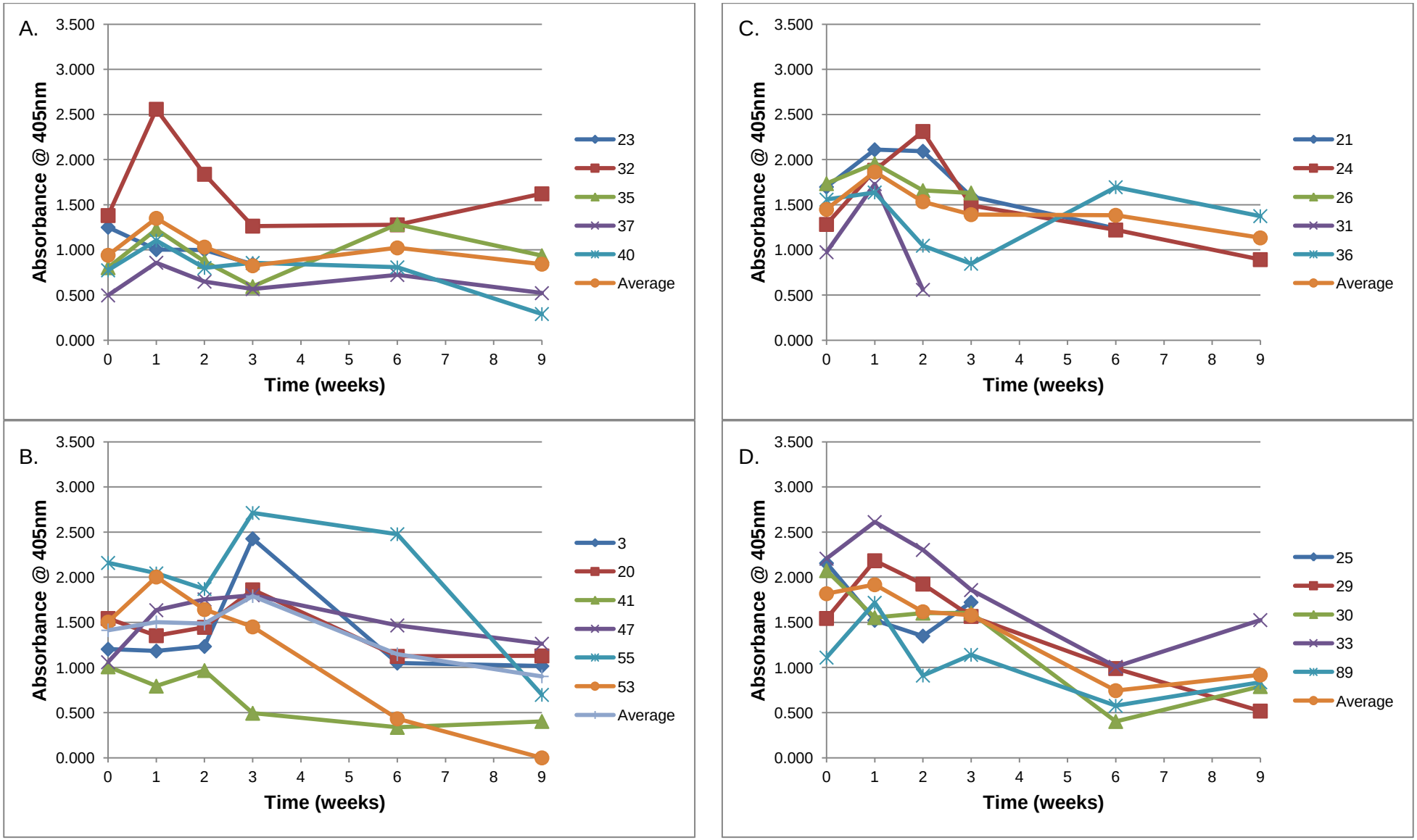

Figure C.11: Humoral immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{13} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. Control group, C. 10^{9} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, D. Received a 10^{10} c.f.u./ml dose of the mucosal VR1020 DNAvaccine.

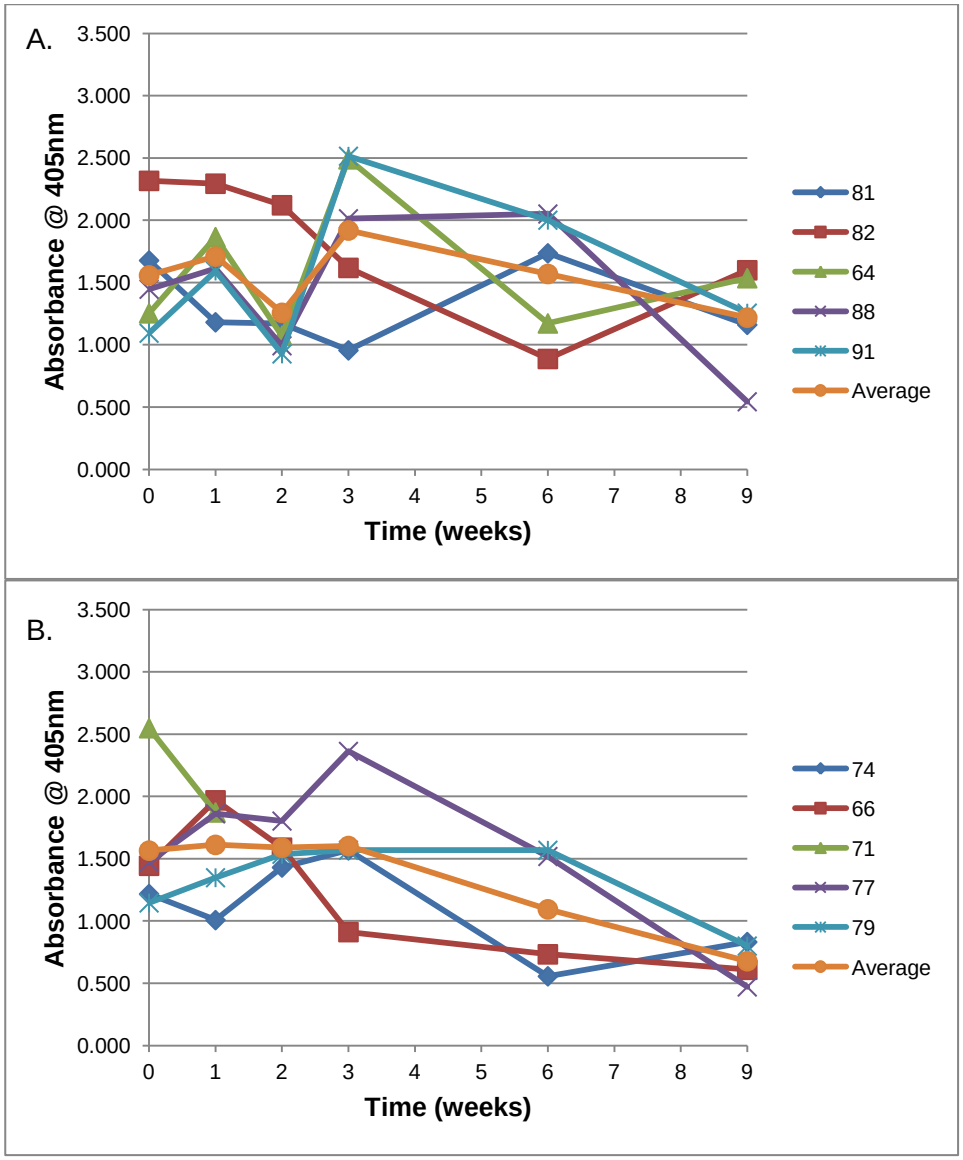

Figure C.12: Humoral immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{11} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, B. 10^{12} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, C. 10^{13} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

Mucosal immune response of ostriches against *Salmonella enterica* serovar *typhimurium* SL3261 LPS

The mucosal immune response of the ostriches against the LPS of *Salmonella enterica* serovar *typhimurium* SL3261 was evaluated using ELISA as described in section 5.2.4. using the secondary antibody rabbit anti-ostrich IgA protein 1. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – LPS – Protein 1
Kromme Rhee
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	329	0.034			
Treatment	10	0.004	0.000	4.45	0.0000
Time	5	0.000	0.000	0.63	0.6805
Treatment x Time	50	0.003	0.000	0.63	0.9744
Residual	264	0.026	0.000		

Grand mean = 0.004 R-squared = 0.2307 C.V. = 229.20%

LSD for Treatment = 0.0051 S.E.D = 0.0026 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00010

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
7	0.01	143.1	1	VR1020 10^9
1	0.01	143.3	2	VR1012 10^9
4	0	134.7	3	VR1012 10^12
2	0	242.1	4	VR1012 10^10
3	0	220.6	5	VR1012 10^11
11	0	198.3	6	VR1020 10^13
6	0	234.1	7	Control
9	0	417	8	VR1020 10^11
5	0	203.4	9	VR1012 10^13
10	0	547.7	10	VR1020 10^12
8	0	380.6	11	VR1020 10^10

LSD for Time = 0.0038 S.E.D = 0.0019 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00010

Time
Averages

Level	--- Y ---	Cv	Rank	Week
6	0.01	233.5	1	9
1	0.01	174.5	2	0
3	0.00	268.4	3	2
5	0.00	249.4	4	6
2	0.00	207.4	5	1
4	0.00	244.6	6	3

LSD for Treatment*Time = 0.0125 S.E.D = 0.0063 r = 5
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00010

Two-way table for Treatment*Time, n=5

	1	2	3	4	5	6
1	0.012	0.014	0.008	0.006	0.006	0.010
2	0.004	0.002	0.002	0.002	0.012	0.002
3	0.002	0.004	0.010	0.004	0.002	0.000
4	0.004	0.004	0.004	0.006	0.004	0.006
5	0.004	0.002	0.000	0.002	0.002	0.002
6	0.008	0.000	0.000	0.002	0.004	0.004
7	0.018	0.006	0.026	0.008	0.010	0.014
8	0.000	0.000	0.000	0.000	0.002	0.002
9	0.002	0.002	0.000	0.000	0.000	0.012
10	0.000	0.000	0.000	0.000	0.000	0.006
11	0.004	0.006	0.004	0.002	0.000	0.004

The following table contains the input data for the ANOVA and is arranged in two columns with the ostrich identification number, treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The treatments consist of VR1012 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (1), $1x10^{10}$ c.f.u./ml (2), $1x10^{11}$ c.f.u./ml (3), $1x10^{12}$ c.f.u./ml (4) and $1x10^{13}$ c.f.u./ml (5); *Salmonella enterica* serovar *typhimurium* SL3261 control (6), VR1020 naked DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (7), $1x10^{10}$ c.f.u./ml (8), $1x10^{11}$ c.f.u./ml (9), $1x10^{12}$ c.f.u./ml (10) and $1x10^{13}$ c.f.u./ml (11). Following the data are the graphs drawn for each ostrich depicting the absorbance measured over time.

Ostrich	Trt	Time	Resp	Weight
2	1	0	0.000	44.6
10	1	0	0.008	47.6
12	1	0	0.016	50.4
18	1	0	0.000	45.2
19	1	0	0.032	47.8
7	2	0	0.012	44.2
9	2	0	0.003	46.8
11	2	0	0.014	48.4
13	2	0	0.000	50
14	2	0	0.000	47
48	3	0	0.001	48.6
56	3	0	0.000	48
57	3	0	0.000	47.6
58	3	0	0.000	47.6
59	3	0	0.012	46.2
44	4	0	0.000	48.2
45	4	0	0.011	46.6
51	4	0	0.013	45.6
52	4	0	0.000	46.8
54	4	0	0.000	51
23	5	0	0.009	51.6
32	5	0	0.000	46.6
35	5	0	0.000	50.4
37	5	0	0.003	49.8
40	5	0	0.009	47.2
3	6	0	0.000	52.4
20	6	0	0.000	52.4
41	6	0	0.000	51.8
47	6	0	0.016	54.8
55	6	0	0.020	55.8
53	6	0	0.003	55.4
21	7	0	0.000	48.8
24	7	0	0.000	50
26	7	0	0.024	52.8
31	7	0	0.041	47
36	7	0	0.031	51.2
25	8	0	0.000	48.2
29	8	0	0.000	46.8
30	8	0	0.000	50
33	8	0	0.000	53.8
89	8	0	0.000	53.2
81	9	0	0.002	44
82	9	0	0.000	50.8
64	9	0	0.000	54
88	9	0	0.000	50.6
91	9	0	0.005	50.8
74	10	0	0.000	55.6
66	10	0	0.000	48.2
71	10	0	0.000	44.8
77	10	0	0.000	49
79	10	0	0.000	44.6
65	11	0	0.014	50.4
69	11	0	0.000	50.6
70	11	0	0.000	47.6
76	11	0	0.004	44
78	11	0	0.008	46.2
2	1	1	0.000	.
10	1	1	0.007	.
12	1	1	0.019	.
18	1	1	0.000	.
19	1	1	0.040	.
7	2	1	0.002	.
9	2	1	.	.
11	2	1	0.003	.
13	2	1	0.006	.
14	2	1	0.000	.
48	3	1	0.004	.
56	3	1	0.012	.
57	3	1	0.000	.
58	3	1	0.000	.
59	3	1	0.014	.
44	4	1	0.000	.
45	4	1	0.005	.
51	4	1	0.008	.
52	4	1	0.000	.
54	4	1	0.000	.
23	5	1	0.006	.
32	5	1	0.000	.
35	5	1	0.000	.
37	5	1	0.004	.
40	5	1	0.001	.
3	6	1	0.000	.
20	6	1	0.000	.
41	6	1	0.000	.
47	6	1	0.000	.
55	6	1	0.000	.
53	6	1	0.003	.
21	7	1	0.016	.
24	7	1	0.000	.
26	7	1	0.000	.
31	7	1	0.000	.
36	7	1	0.008	.
25	8	1	0.000	.
29	8	1	0.000	.
30	8	1	0.000	.
33	8	1	0.000	.
89	8	1	0.004	.
81	9	1	0.008	.
82	9	1	0.000	.
64	9	1	0.000	.
88	9	1	0.000	.
91	9	1	0.000	.
74	10	1	0.000	.
66	10	1	0.000	.
71	10	1	0.000	.
77	10	1	0.000	.
79	10	1	0.000	.
65	11	1	.	.
69	11	1	0.000	.
70	11	1	0.002	.
76	11	1	0.006	.
78	11	1	0.019	.
2	1	2	0.000	41.8
10	1	2	0.002	51.5
12	1	2	0.039	47
18	1	2	0.000	40.2
19	1	2	0.000	36.2
7	2	2	0.000	43.2
9	2	2	.	.
11	2	2	0.009	44.4
13	2	2	0.000	44.4
14	2	2	0.000	40.6
48	3	2	0.038	47
56	3	2	0.000	40.6
57	3	2	0.000	44.2

58	3	2	0.000	36
59	3	2	0.013	36.6
44	4	2	0.000	40
45	4	2	0.008	39.8
51	4	2	0.012	40
52	4	2	0.000	58.5
54	4	2	0.000	42
23	5	2	0.000	47.6
32	5	2	0.000	45
35	5	2	0.000	47.8
37	5	2	0.000	47
40	5	2	0.003	41.4
3	6	2	0.000	53
20	6	2	0.000	47.4
41	6	2	0.000	41.2
47	6	2	0.000	47
55	6	2	0.000	50.5
53	6	2	0.036	47.2
21	7	2	0.071	51
24	7	2	0.000	55.5
26	7	2	0.028	42.8
31	7	2	0.000	35
36	7	2	0.028	52.5
25	8	2	0.000	34.6
29	8	2	0.000	45
30	8	2	0.000	48.2
33	8	2	0.000	51
89	8	2	0.000	54
81	9	2	0.000	46.8
82	9	2	0.000	46.2
64	9	2	0.000	45.8
88	9	2	0.000	52.5
91	9	2	0.000	37.2
74	10	2	0.000	49
66	10	2	0.000	42.6
71	10	2	.	.
77	10	2	0.000	39.2
79	10	2	0.000	40.4
65	11	2	.	.
69	11	2	0.000	40.6
70	11	2	0.000	41.4
76	11	2	0.015	36.2
78	11	2	.	.
2	1	3	0.000	.
10	1	3	0.017	.
12	1	3	0.000	.
18	1	3	0.000	.
19	1	3	0.009	.
7	2	3	0.000	.
9	2	3	.	.
11	2	3	0.011	.
13	2	3	0.002	.
14	2	3	0.000	.
48	3	3	0.005	.
56	3	3	0.000	.
57	3	3	0.000	.
58	3	3	0.000	.
59	3	3	0.014	.
44	4	3	0.000	.
45	4	3	0.009	.
51	4	3	0.017	.
52	4	3	0.000	.
54	4	3	0.000	.

23	5	3	0.000	.
32	5	3	0.000	.
35	5	3	0.000	.
37	5	3	0.006	.
40	5	3	0.001	.
3	6	3	0.000	.
20	6	3	0.000	.
41	6	3	0.000	.
47	6	3	0.000	.
55	6	3	0.011	.
53	6	3	0.023	.
21	7	3	0.000	.
24	7	3	0.000	.
26	7	3	0.000	.
31	7	3	.	.
36	7	3	0.038	.
25	8	3	0.000	.
29	8	3	0.000	.
30	8	3	0.000	.
33	8	3	0.000	.
89	8	3	0.000	.
81	9	3	0.004	.
82	9	3	0.000	.
64	9	3	0.000	.
88	9	3	0.000	.
91	9	3	0.000	.
74	10	3	0.000	.
66	10	3	0.000	.
71	10	3	.	.
77	10	3	0.000	.
79	10	3	0.000	.
65	11	3	.	.
69	11	3	0.000	.
70	11	3	0.002	.
76	11	3	0.010	.
78	11	3	.	.
2	1	6	0.000	.
10	1	6	0.000	.
12	1	6	0.031	.
18	1	6	0.000	.
19	1	6	.	.
7	2	6	0.052	.
9	2	6	.	.
11	2	6	0.010	.
13	2	6	0.000	.
14	2	6	0.000	.
48	3	6	0.000	.
56	3	6	0.000	.
57	3	6	0.000	.
58	3	6	0.000	.
59	3	6	0.007	.
44	4	6	0.000	.
45	4	6	0.008	.
51	4	6	0.012	.
52	4	6	0.000	.
54	4	6	0.000	.
23	5	6	.	.
32	5	6	0.008	.
35	5	6	0.000	.
37	5	6	0.002	.
40	5	6	0.000	.
3	6	6	0.000	.
20	6	6	0.000	.

41	6	6	0.000	.
47	6	6	0.000	.
55	6	6	0.018	.
53	6	6	0.027	.
21	7	6	0.032	.
24	7	6	0.000	.
26	7	6	.	.
31	7	6	.	.
36	7	6	0.015	.
25	8	6	.	.
29	8	6	0.000	.
30	8	6	0.000	.
33	8	6	0.000	.
89	8	6	0.011	.
81	9	6	0.002	.
82	9	6	0.000	.
64	9	6	0.000	.
88	9	6	0.000	.
91	9	6	0.000	.
74	10	6	0.001	.
66	10	6	0.000	.
71	10	6	.	.
77	10	6	0.000	.
79	10	6	0.000	.
65	11	6	.	.
69	11	6	0.000	.
70	11	6	0.003	.
76	11	6	0.001	.
78	11	6	.	.
2	1	9	0.000	48
10	1	9	0.000	59
12	1	9	0.030	47.8
18	1	9	0.017	46.6
19	1	9	.	.
7	2	9	0.013	39
9	2	9	.	.
11	2	9	0.000	63
13	2	9	.	.
14	2	9	0.001	53
48	3	9	0.000	53
56	3	9	0.000	44.4
57	3	9	0.000	44.4
58	3	9	0.000	48
59	3	9	.	.
44	4	9	0.000	39.2
45	4	9	0.008	51.5
51	4	9	0.015	39.4
52	4	9	0.000	61
54	4	9	0.001	38.4
23	5	9	.	.
32	5	9	0.000	49.8
35	5	9	0.000	53
37	5	9	0.004	48.4
40	5	9	0.005	61.5
3	6	9	0.000	44.8
20	6	9	0.000	38.6
41	6	9	0.000	55
47	6	9	0.018	64
55	6	9	0.000	48.4
53	6	9	.	.
21	7	9	.	.
24	7	9	0.009	63
26	7	9	.	.
31	7	9	.	.
36	7	9	0.063	60
25	8	9	.	.
29	8	9	0.000	59
30	8	9	0.000	54
33	8	9	0.000	60.5
89	8	9	0.011	59.5
81	9	9	0.000	43.4
82	9	9	0.000	57.5
64	9	9	0.000	42.6
88	9	9	0.057	53
91	9	9	0.000	52.5
74	10	9	0.030	57.5
66	10	9	0.000	62.5
71	10	9	.	.
77	10	9	0.000	39
79	10	9	0.000	43.6
65	11	9	.	.
69	11	9	0.000	52
70	11	9	0.003	54
76	11	9	0.018	33
78	11	9	.	.

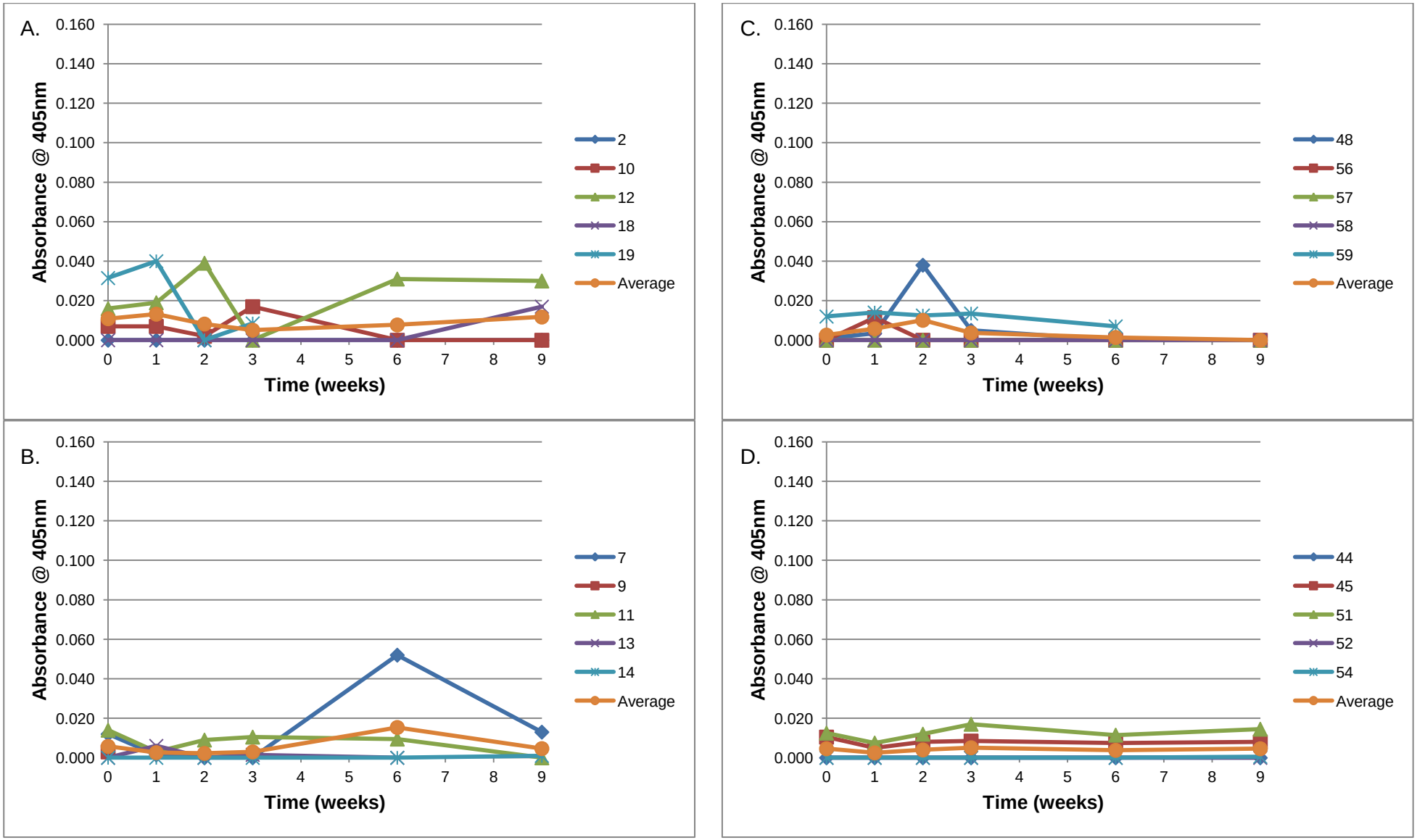

Figure C.13: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^9 c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. 10^{10} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, C. 10^{11} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, D. Received a 10^{12} c.f.u./ml dose of the VR1012 mucosal DNA vaccine.

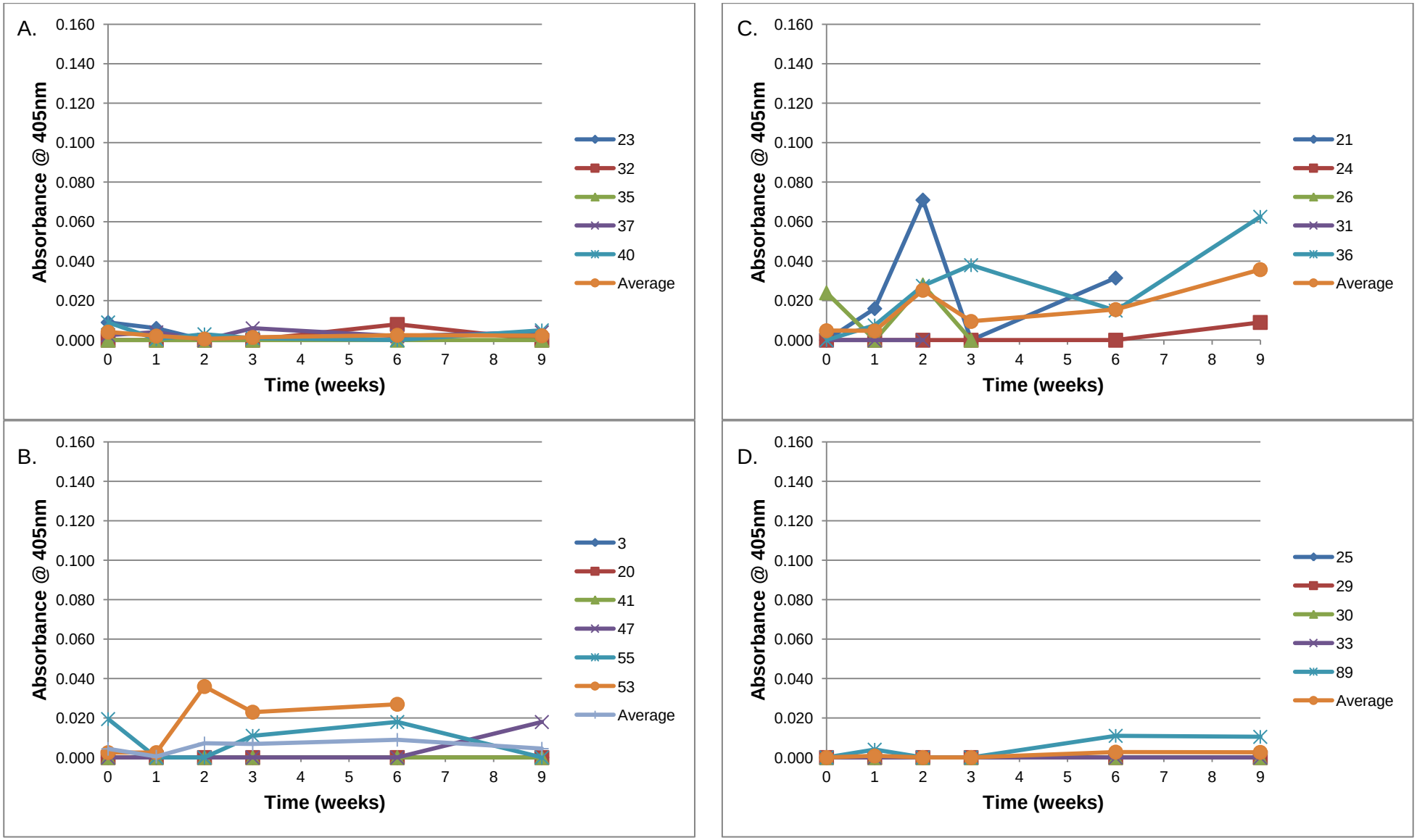

Figure C.14: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{13} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. Control group, C. 10^{9} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, D. Received a 10^{10} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

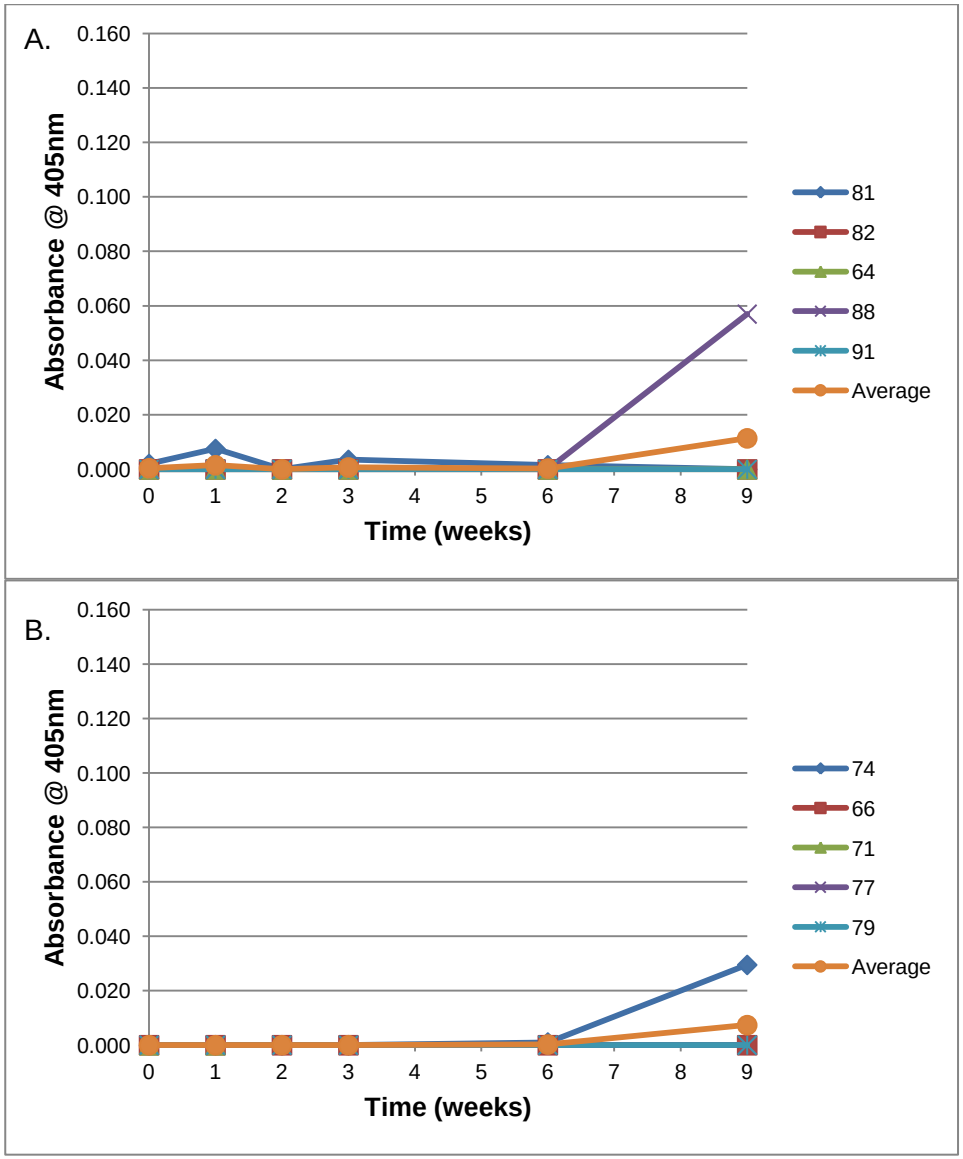

Figure C.15: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{11} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, B. 10^{12} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, C. 10^{13} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

Mucosal immune response of ostriches against *Salmonella enterica* serovar *typhimurium* SL3261 LPS

The mucosal immune response of the ostriches against the LPS of *Salmonella enterica* serovar *typhimurium* SL3261 was evaluated using ELISA as described in section 5.2.4., using the secondary antibody rabbit anti-ostrich IgA protein 2. An ANOVA was performed using the Statistical Analysis program Agrobase Generation II® (Agronomix Software Inc.).

A N A L Y S I S O F V A R I A N C E
Swab – LPS – Protein 2
Kromme Rhee
Variable: RESP

Source	df	SS	MS	F-value	Pr>F
Total	329	0.083			
Treatment	10	0.011	0.001	4.73	0.0000
Time	5	0.001	0.000	1.07	0.3746
Treatment x Time	50	0.011	0.000	0.94	0.5942
Residual	264	0.060	0.000		

Grand mean = 0.006 R-squared = 0.2740 C.V. = 251.7%

LSD for Treatment = 0.0077 S.E.D = 0.0039 r = 30
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00023

Treatment
Averages

Level	--- Y ---	Cv	Rank	Group
9	0.02	163.5	1	VR1020 10^11
6	0.01	205.9	2	Control
2	0.01	163.2	3	VR1012 10^10
7	0.01	178.3	4	VR1020 10^9
4	0.01	285.7	5	VR1012 10^12
5	0.01	136.5	6	VR1012 10^13
11	0.00	242.7	7	VR1020 10^13
1	0.00	276.7	8	VR1012 10^9
8	0.00	276.7	9	VR1020 10^10
3	0.00	331.7	10	VR1012 10^11
10	0.00	-9.0	11	VR1020 10^12

LSD for Time = 0.0057 S.E.D = 0.0029 r = 55
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00023

Time
Averages

Level	--- Y ---	Cv	Rank	Week
2	0.01	256.2	1	1
1	0.01	218.9	2	0
6	0.01	278.4	3	9
5	0.01	231.0	4	6
3	0.00	304.5	5	2
4	0.00	248.1	6	3

LSD for Treatment*Time = 0.0188 S.E.D = 0.0096 r = 5
t (2-sided a=0.050, 264 df) = 1.9690 MSE = 0.00023

Two-way table for Treatment*Time, n=5

	1	2	3	4	5	6
1	0.012	0.014	0.008	0.006	0.006	0.010
2	0.004	0.002	0.002	0.002	0.012	0.002
3	0.002	0.004	0.010	0.004	0.002	0.000
4	0.004	0.004	0.004	0.006	0.004	0.006
5	0.004	0.002	0.000	0.002	0.002	0.002
6	0.008	0.000	0.000	0.002	0.004	0.004
7	0.018	0.006	0.026	0.008	0.010	0.014
8	0.000	0.000	0.000	0.000	0.002	0.002
9	0.002	0.002	0.000	0.000	0.000	0.012
10	0.000	0.000	0.000	0.000	0.000	0.006
11	0.004	0.006	0.004	0.002	0.000	0.004

The following table contains the input data for the ANOVA and is arranged in two columns with the ostrich identification number, treatment received (Trt), Time in weeks, the response as measured by ELISA and weights of the ostriches, read from the top to the bottom and left to right. The treatments consist of VR1012 mucosal DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (1), $1x10^{10}$ c.f.u./ml (2), $1x10^{11}$ c.f.u./ml (3), $1x10^{12}$ c.f.u./ml (4) and $1x10^{13}$ c.f.u./ml (5); *Salmonella enterica* serovar *typhimurium* SL3261 control (6), VR1020 naked DNA vaccine administered at various doses; $1x10^{9}$ c.f.u./ml (7), $1x10^{10}$ c.f.u./ml (8), $1x10^{11}$ c.f.u./ml (9), $1x10^{12}$ c.f.u./ml (10) and $1x10^{13}$ c.f.u./ml (11). Following the data are the graphs drawn for each ostrich depicting the absorbance measured over time.

Ostrich	Trt	Time	Resp	Weight
2	1	0	0.000	44.6
10	1	0	0.008	47.6
12	1	0	0.000	50.4
18	1	0	0.035	45.2
19	1	0	0.000	47.8
7	2	0	0.000	44.2
9	2	0	0.000	46.8
11	2	0	0.040	48.4
13	2	0	0.014	50
14	2	0	0.013	47
48	3	0	0.000	48.6
56	3	0	0.000	48
57	3	0	0.000	47.6
58	3	0	0.000	47.6
59	3	0	0.014	46.2
44	4	0	0.000	48.2
45	4	0	0.009	46.6
51	4	0	0.000	45.6
52	4	0	0.000	46.8
54	4	0	0.000	51
23	5	0	0.000	51.6
32	5	0	0.005	46.6
35	5	0	0.007	50.4
37	5	0	0.000	49.8
40	5	0	0.010	47.2
3	6	0	0.000	52.4
20	6	0	0.000	52.4
41	6	0	0.000	51.8
47	6	0	0.029	54.8
55	6	0	0.009	55.8
53	6	0	0.003	55.4
21	7	0	0.003	48.8
24	7	0	0.000	50
26	7	0	0.019	52.8
31	7	0	0.006	47
36	7	0	0.024	51.2
25	8	0	0.004	48.2
29	8	0	0.004	46.8
30	8	0	0.000	50
33	8	0	0.002	53.8
89	8	0	0.000	53.2
81	9	0	0.004	44
82	9	0	0.101	50.8
64	9	0	0.013	54
88	9	0	0.008	50.6
91	9	0	0.001	50.8
74	10	0	0.000	55.6
66	10	0	0.000	48.2
71	10	0	0.000	44.8
77	10	0	0.000	49
79	10	0	0.000	44.6
65	11	0	0.003	50.4
69	11	0	0.000	50.6
70	11	0	0.006	47.6
76	11	0	0.000	44
78	11	0	0.024	46.2
2	1	1	0.000	.
10	1	1	0.000	.
12	1	1	0.000	.
18	1	1	0.000	.
19	1	1	0.000	.
7	2	1	0.022	.
9	2	1	.	.
11	2	1	0.001	.
13	2	1	0.012	.
14	2	1	0.016	.
48	3	1	0.000	.
56	3	1	0.000	.
57	3	1	0.000	.
58	3	1	0.000	.
59	3	1	0.005	.
44	4	1	0.000	.
45	4	1	0.000	.
51	4	1	0.000	.
52	4	1	0.084	.
54	4	1	0.000	.
23	5	1	0.000	.
32	5	1	0.007	.
35	5	1	0.000	.
37	5	1	0.003	.
40	5	1	0.003	.
3	6	1	0.000	.
20	6	1	0.000	.
41	6	1	0.000	.
47	6	1	0.021	.
55	6	1	0.004	.
53	6	1	0.000	.
21	7	1	0.000	.
24	7	1	0.008	.
26	7	1	0.000	.
31	7	1	0.040	.
36	7	1	0.000	.
25	8	1	0.006	.
29	8	1	0.000	.
30	8	1	0.000	.
33	8	1	0.000	.
89	8	1	0.000	.
81	9	1	0.014	.
82	9	1	0.106	.
64	9	1	0.081	.
88	9	1	0.000	.
91	9	1	0.000	.
74	10	1	0.000	.
66	10	1	0.000	.
71	10	1	0.000	.
77	10	1	0.000	.
79	10	1	0.000	.
65	11	1	.	.
69	11	1	0.000	.
70	11	1	0.000	.
76	11	1	0.000	.
78	11	1	0.041	.
2	1	2	0.000	41.8
10	1	2	0.000	51.5
12	1	2	0.000	47
18	1	2	0.000	40.2
19	1	2	0.003	36.2
7	2	2	0.011	43.2
9	2	2	.	.
11	2	2	0.003	44.4
13	2	2	0.004	44.4

14	2	2	0.002	40.6
48	3	2	0.000	47
56	3	2	0.000	40.6
57	3	2	0.000	44.2
58	3	2	0.000	36
59	3	2	0.001	36.6
44	4	2	0.000	40
45	4	2	0.001	39.8
51	4	2	0.000	40
52	4	2	0.000	58.5
54	4	2	0.000	42
23	5	2	0.000	47.6
32	5	2	0.005	45
35	5	2	0.000	47.8
37	5	2	0.007	47
40	5	2	0.007	41.4
3	6	2	0.000	53
20	6	2	0.000	47.4
41	6	2	0.000	41.2
47	6	2	0.000	47
55	6	2	0.000	50.5
53	6	2	0.006	47.2
21	7	2	0.004	51
24	7	2	0.000	55.5
26	7	2	0.025	42.8
31	7	2	0.026	35
36	7	2	0.011	52.5
25	8	2	0.000	34.6
29	8	2	0.000	45
30	8	2	0.000	48.2
33	8	2	0.000	51
89	8	2	0.001	54
81	9	2	0.033	46.8
82	9	2	0.067	46.2
64	9	2	0.000	45.8
88	9	2	0.000	52.5
91	9	2	0.000	37.2
74	10	2	0.000	49
66	10	2	0.000	42.6
71	10	2	.	.
77	10	2	0.000	39.2
79	10	2	0.000	40.4
65	11	2	.	.
69	11	2	0.000	40.6
70	11	2	0.002	41.4
76	11	2	0.000	36.2
78	11	2	.	.
2	1	3	0.000	.
10	1	3	0.000	.
12	1	3	0.000	.
18	1	3	0.005	.
19	1	3	0.000	.
7	2	3	0.000	.
9	2	3	.	.
11	2	3	0.000	.
13	2	3	0.008	.
14	2	3	0.000	.
48	3	3	0.000	.
56	3	3	0.000	.
57	3	3	0.001	.
58	3	3	0.000	.
59	3	3	0.007	.
44	4	3	0.000	.
45	4	3	0.000	.
51	4	3	0.006	.
52	4	3	0.000	.
54	4	3	0.000	.
23	5	3	0.000	.
32	5	3	0.025	.
35	5	3	0.000	.
37	5	3	0.005	.
40	5	3	0.001	.
3	6	3	0.000	.
20	6	3	0.000	.
41	6	3	0.000	.
47	6	3	0.026	.
55	6	3	0.000	.
53	6	3	0.015	.
21	7	3	0.000	.
24	7	3	0.000	.
26	7	3	0.000	.
31	7	3	.	.
36	7	3	0.019	.
25	8	3	0.000	.
29	8	3	0.000	.
30	8	3	0.000	.
33	8	3	0.004	.
89	8	3	0.028	.
81	9	3	0.000	.
82	9	3	0.000	.
64	9	3	0.000	.
88	9	3	0.000	.
91	9	3	0.000	.
74	10	3	0.000	.
66	10	3	0.000	.
71	10	3	.	.
77	10	3	0.000	.
79	10	3	0.000	.
65	11	3	.	.
69	11	3	0.000	.
70	11	3	0.000	.
76	11	3	0.008	.
78	11	3	.	.
2	1	6	0.000	.
10	1	6	0.004	.
12	1	6	0.000	.
18	1	6	0.000	.
19	1	6	.	.
7	2	6	0.014	.
9	2	6	.	.
11	2	6	0.000	.
13	2	6	0.028	.
14	2	6	0.000	.
48	3	6	0.000	.
56	3	6	0.004	.
57	3	6	0.000	.
58	3	6	0.000	.
59	3	6	0.043	.
44	4	6	0.000	.
45	4	6	0.045	.
51	4	6	0.015	.
52	4	6	0.000	.

54	4	6	0.000	.
23	5	6	.	.
32	5	6	0.000	.
35	5	6	0.008	.
37	5	6	0.000	.
40	5	6	0.000	.
3	6	6	0.000	.
20	6	6	0.000	.
41	6	6	0.000	.
47	6	6	0.018	.
55	6	6	0.055	.
53	6	6	0.043	.
21	7	6	0.002	.
24	7	6	0.000	.
26	7	6	.	.
31	7	6	.	.
36	7	6	0.000	.
25	8	6	.	.
29	8	6	0.000	.
30	8	6	0.000	.
33	8	6	0.041	.
89	8	6	0.006	.
81	9	6	0.004	.
82	9	6	0.000	.
64	9	6	0.054	.
88	9	6	0.000	.
91	9	6	0.000	.
74	10	6	0.000	.
66	10	6	0.000	.
71	10	6	.	.
77	10	6	0.000	.
79	10	6	0.000	.
65	11	6	.	.
69	11	6	0.000	.
70	11	6	0.008	.
76	11	6	0.000	.
78	11	6	.	.
2	1	9	0.011	48
10	1	9	0.000	59
12	1	9	0.000	47.8
18	1	9	0.029	46.6
19	1	9	.	.
7	2	9	0.022	39
9	2	9	.	.
11	2	9	0.000	63
13	2	9	.	.
14	2	9	0.004	53
48	3	9	0.000	53
56	3	9	0.001	44.4
57	3	9	0.000	44.4
58	3	9	0.000	48
59	3	9	.	.
44	4	9	0.000	39.2
45	4	9	0.000	51.5
51	4	9	0.008	39.4
52	4	9	0.000	61
54	4	9	0.000	38.4
23	5	9	.	.
32	5	9	0.008	49.8
35	5	9	0.006	53
37	5	9	0.000	48.4
40	5	9	0.013	61.5
3	6	9	0.000	44.8
20	6	9	0.000	38.6
41	6	9	0.000	55
47	6	9	0.013	64
55	6	9	0.021	48.4
53	6	9	.	.
21	7	9	.	.
24	7	9	0.000	63
26	7	9	.	.
31	7	9	.	.
36	7	9	0.000	60
25	8	9	.	.
29	8	9	0.000	59
30	8	9	0.000	54
33	8	9	0.001	60.5
89	8	9	0.006	59.5
81	9	9	0.007	43.4
82	9	9	0.119	57.5
64	9	9	0.071	42.6
88	9	9	0.023	53
91	9	9	0.000	52.5
74	10	9	0.000	57.5
66	10	9	0.000	62.5
71	10	9	.	.
77	10	9	0.000	39
79	10	9	0.000	43.6
65	11	9	.	.
69	11	9	0.015	52
70	11	9	0.000	54
76	11	9	0.003	33
78	11	9	.	.

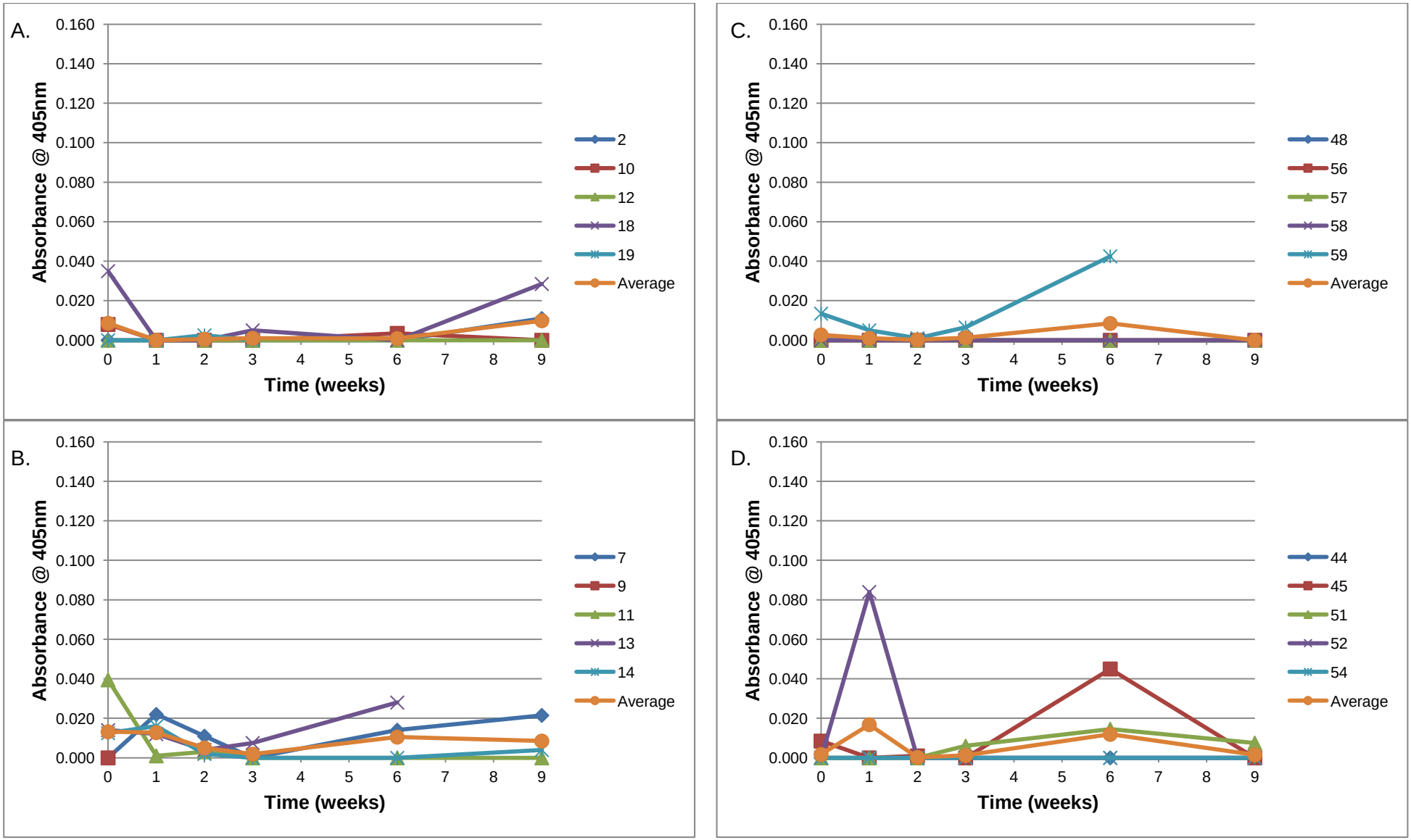

Figure C.16: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^9 c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. 10^{10} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, C. 10^{11} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, D. Received a 10^{12} c.f.u./ml dose of the VR1012 mucosal DNA vaccine.

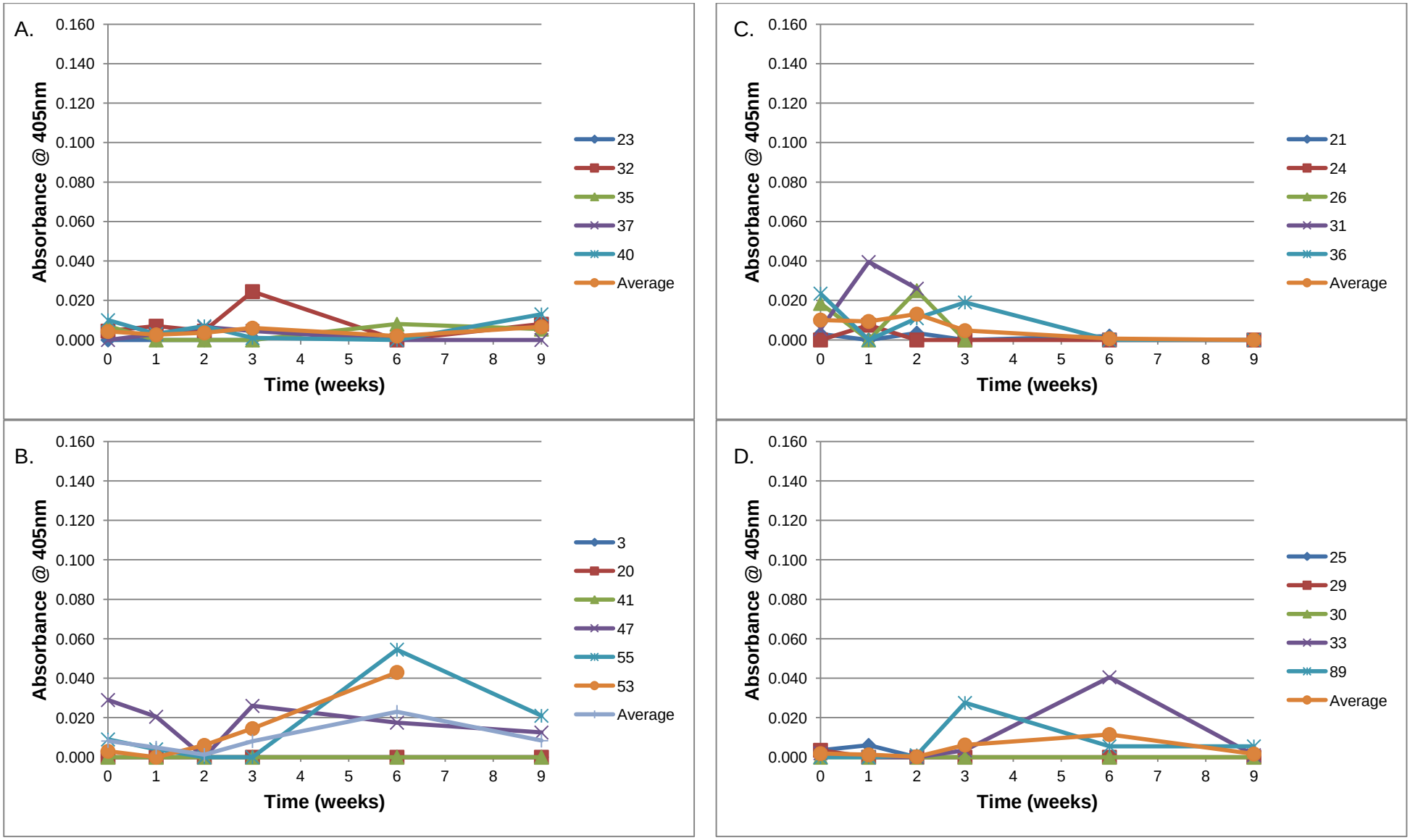

Figure C.17: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{13} c.f.u./ml dose of the VR1012 mucosal DNA vaccine, B. Control group, C. 10^{9} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, D. Received a 10^{10} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.

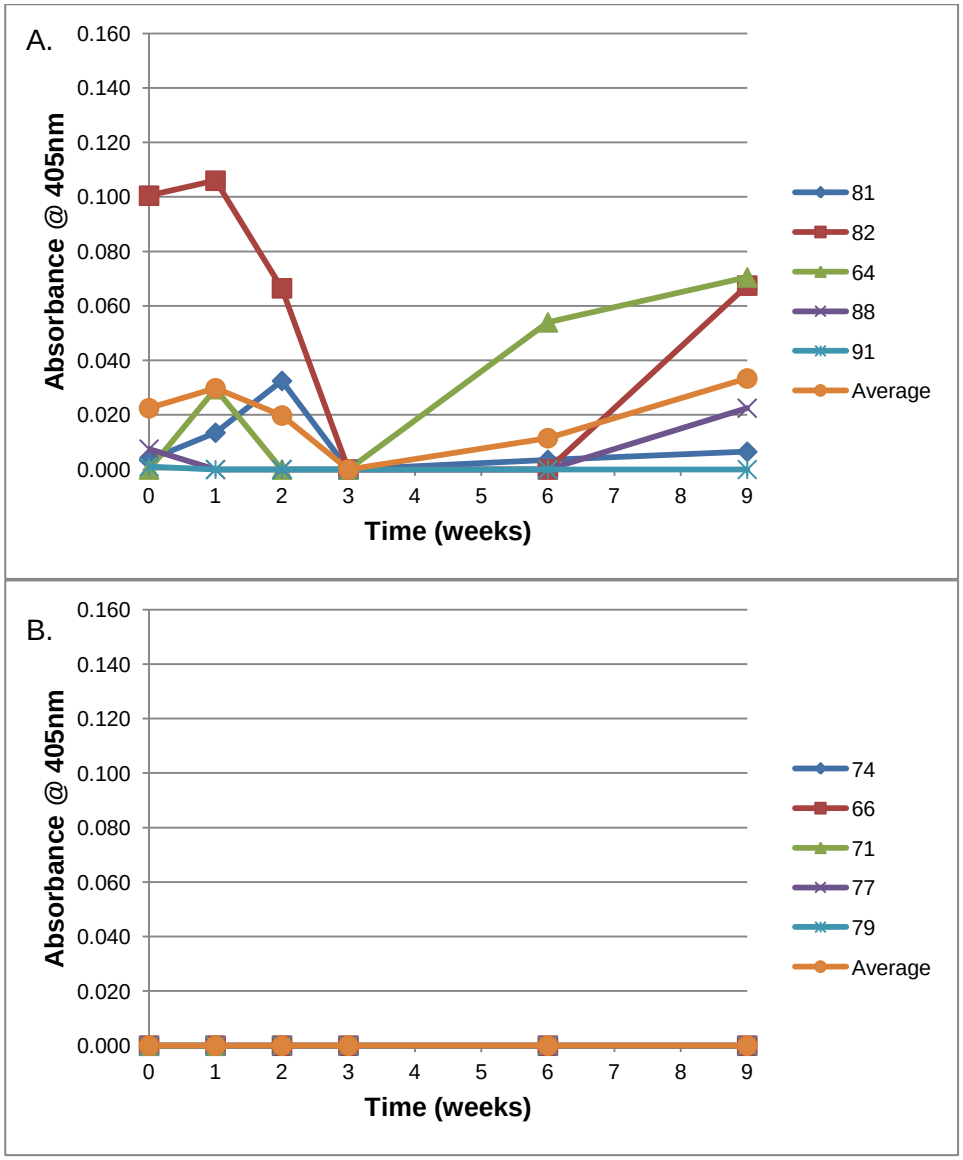

Figure C.18: Mucosal immune responses elicited in ostriches during the dosing vaccine trial at Kromme Rhee farm in Stellenbosch, Western Cape. Various doses of the mucosal vaccines were evaluated. A. 10^{11} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, B. 10^{12} c.f.u./ml dose of the VR1020 mucosal DNA vaccine, C. 10^{13} c.f.u./ml dose of the VR1020 mucosal DNA vaccine.